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(54) **Title** : RECOMBINANT HUMAN IMMUNODEFICIENCY VIRUS VECTOR AND PROCESS FOR PRODUCING THE SAME

(54) 発明の名称 組換えヒト免疫不全ウイルスベクターとその製造方法

(57) Abstract

A recombinant HIV vector which retains the foreign DNA sequence antagonistic against HIV growth and/or gene expression and wherein the U3 domain of the LTR secuence on the genome 5' side has been replaced by the promoter sequence of a cytomegalovirus. As this vector can express an anti-HIV sequence of, for example, antisense, ribozyme or TAR decoy in HIV-infected cells without fail, it is useful as a material for gene therapy of HIV infection.

（５７）要約

この発明は、ＨＩＶの増殖および／または遺伝子発現に対して拮抗的に作用する外来性ＤＮＡ配列を保持するとともに、ゲノム５’側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されている組換えＨＩＶベクターを提供する。

この発明の組換えＨＩＶベクターは、アンチセンス、リボザイム、ＴＡＲデコイ等の抗ＨＩＶ配列をＨＩＶ感染細胞て確実に発現するため、ＨＩＶ感染症に対する遺伝子治療の材料として有用である。

組換えヒト免疫不全ウイルスベクターとその製造方法

技術分野

この発明は、組換えヒト免疫不全ウイルスベクターとその製造方法に関するものである。さらに詳しくは、この発明は、ヒト免疫不全ウイルス（以下、ＨＩＶと略記する）の感染症に対する遺伝子治療等に有用な新しい組換えＨＩＶベクターとその製造方法に関するものである。

背景技術

近年の細胞への遺伝子導入技術の進歩に伴い、これを遺伝子治療に応用しようとする試みがさかんになされるようになってきた。遺伝子治療とは、疾患の原因遺伝子に対して拮抗的に作用する組換え遺伝子を体内に導入して発現させることで疾患の治療を行なう方法であり、米国においては既にアデノシンデアミナーゼ（ＡＤＡ）欠損症、低密度リポ蛋白質受容体欠損症、あるいは嚢胞性線維症等の遺伝病、脳腫瘍や悪性黒色腫等の癌に対する遺伝子治療が開始されている。また最近では、ＡＩＤＳをはじめとしたウイルス感染症に対する遺伝子治療の基礎的検討も数多くなされている。

このような遺伝子治療において現在行なわれている遺伝子導入法は、マウスのレトロウイルスであるMolony Murine Leukemia Virus （以下、ＭｏＭＬＶと略記する）

をベクターとして用いる方法が主流となっている。このＭｏＭＬＶベクターを用いる利点は、多くの種類の細胞に対して遺伝子導入が可能なことであり、遺伝子治療の対象となる種々の疾患に対して広く用いることができる。しかし、静脈内投与や筋肉内投与等のようなin vivoでの遺伝子導入の場合には、組織特異性を持たないというこのベクターの本質的な性質が逆に欠点となる。つまりこのＭｏＭＬＶベクターを患者に直接投与しても、投与部位近傍の細胞にベクターがトラップされてしまい標的細胞に対して遺伝子を導入することが困難となる。また、標的細胞以外の細胞に対して組換え遺伝子が導入されれば副作用の原因ともなりうる。このような理由からＭｏＭＬＶベクターを用いた現在の遺伝子治療の基本的なプロトコールは、自家移植による方法、すなわち患者から標的細胞を体外に取り出して培養を行ない、in vitroでベクターを導入し、その後その細胞を再び患者に戻す方法が採えられている。しかしこの方法には、①細胞の種類によっては生体外に取り出すのが困難なことや標的細胞のみを選別するために手間がかかること、②無菌的な培養をするための特殊な設備が必要であり、治療を実施できる施設が限定されること等の問題が残されている。

これらの問題を解決するための一つの方法として組織特異的なウイルスベクターの使用が検討されている。すなわち、ヒトのレトロウイルスであるＨＩＶはウイルスエンベロープ蛋白であるｇｐ１２０がヒトのＣＤ４陽性Ｔ細胞表

面に存在するＣＤ４分子と結合して感染する。そこでこの特異的な感染機構を利用したＨＩＶベクターという新しい組換えウイルスベクターが提案されている（Shimada,T., et al., J.Clin. Invest., 88, 1043, 1991）。ＨＩＶベクターの最大の特徴はＣＤ４陽性Ｔ細胞に対して高い効率で特異的に外来性遺伝子を導入することができる点である。この組織特異的ベクターは直接患者に投与してin vivoで標的細胞に遺伝子を導入することもできることから、特殊な設備を必要とせず、普通の外来治療として治療行為を行なうことも可能となり、ＣＤ４陽性Ｔ細胞を標的細胞とした今後の遺伝子治療に大きく貢献するものと期待されている。

遺伝子治療によって治療効果が期待できる疾患は、先天性、後天性を問わず遺伝子の異常が原因で発症する疾患全てが含まれる。特に、致死的であり、かつ治療法が確立されていない癌や後天性免疫不全症候群（ＡＩＤＳ）等の疾患に対しては非常に有用性の高い治療方法であると考えられる。

とりわけ、ＡＩＤＳはＨＩＶに感染することにより免疫不全状態となり、種々の日和見感染症を発症して最終的には確実に死に至る疾患である。ＡＩＤＳに対する現在の治療薬としてはアジドチミジン（ＡＺＴ）、ジダノシン（ｄｄＩ）、ザルシタビン（ｄｄＣ）等のヌクレオシド系逆転写酵素阻害剤が使用されている。しかし、これらの薬剤は既に感染した細胞を除去する作用はなく、その上重篤な

副作用がみられることや耐性ウイルスの出現により薬剤が無効となる例がある等の問題があり、新しい治療薬の開発が強く望まれている。その候補の一つとして、ＨＩＶの増殖および／または遺伝子発現に対して拮抗的に作用するＤＮＡやＲＮＡ等の遺伝高分子配列（以下、抗ＨＩＶ配列と記載することがある）が注目されている。すなわち、ＨＩＶゲノムの特定遺伝子領域に対するアンチセンス鎖、リボザイム、デコイ等である。アンチセンスはゲノム配列に相補的な配列をもつＤＮＡまたはＲＮＡ分子で、細胞内でウイルスに由来する遺伝子と部分的に二重鎖を作ることによりウイルスの増殖を阻害したり、遺伝子発現を抑制すると考えられている。Matsukura らは化学的に合成されたアンチセンスＤＮＡを培地に添加することにより、培養細胞でのＨＩＶの増殖を抑制できることを報告している（Proc. Natl. Acad. Sci. U.S.A., 86, 4244-4248, 1989）。リボザイムはＲＮＡを切断する酵素様の活性をもつＲＮＡ分子であり、特に、基質と相補的に結合する部分を任意の配列に変えることができるハンマーヘッド型リボザイムやヘアピン型リボザイムは抗ウイルス剤としての有用性が期待されている（J. Rossi, et al., Pharmac. Ther. 50, 245-254, 1991;M. Yu. et al., Proc. Natl, Acad. Sci. U.S.A., 90, 6340-6344, 1993）。ＴＡＲデコイはtrans-acting factor とcis-acting elementとの結合を競合分子によって競合阻害しようという考え方に基づくものである。すなわち、ＨＩＶではその発現産物ＴａｔがｍＲＮＡのＴ

ＡＲ配列に結合することが遺伝子発現にとって必須であると考えられている。このＴＡＲ配列をもつＲＮＡ分子（ＴＡＲデコイ）が細胞内に存在するとＴａｔはこのＴＡＲデコイとの結合に使われてしまいｍＲＮＡとの結合ができなくなるためにＨＩＶが増殖できなくなる。

しかしながら、以上に述べた抗ＨＩＶ配列はＤＮＡ合成装置を用いて簡単に調製することができるという利点があるが、その反面、これらを実際に治療に使うためには、その安定性、溶解度、吸収率などにまだ多くの改良が必要であることや、合成のための費用が高すぎるなどの問題点がある。

また別の試みとして、これら抗ＨＩＶ配列を細胞内で発現させるようにデザインしたプラスミドをリン酸カルシウムやカチオン性脂質を用いた方法、マイクロインジェクションによる方法、あるいはＭｏＭＬＶベクターを用いた方法でＨＩＶ感染細胞に導入し、ＨＩＶの増殖を抑制しようとする遺伝子治療の基礎的検討も数多くなされている（G. Sczakiel, et.al., Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, 179-193, 1991; Sullenger, B.A., et.al., 63, 601-608, 1990; N. Sarver, et. al., Science, 247, 1222-1225, 1990; A. Rhodes and W. James, J. General Virology, 71, 1965-1974, 1990）。これらの報告においては、細胞内で抗ＨＩＶ配列が発現すること、およびこれらの配列の発現によって細胞に感染したＨＩＶの増殖が抑制されたことが証明されてい

る。しかしながら、このようなプラスミドベクターを用いた遺伝子導入方法では、in vivoで標的となる細胞へ特異的に遺伝子を導入することが困難であるばかりか、細胞に対する毒性が強いこと、遺伝子導入操作が煩雑であること、導入された遺伝子の染色体への組み込みがほとんど起こらないために抗ウイルス遺伝子の発現が一過性であること等の大きな問題があり、このままの形で治療に応用することは不可能である。

以上のような理由から、ヒトのＨＩＶ感染細胞に抗ＨＩＶ配列を導入するための最も適した手段として、ＨＩＶそのものをベクターとして用いる遺伝子治療法が提案されている。すなわち、このＨＩＶベクターを用いることによって、ＨＩＶに感染したＣＤ４陽性Ｔ細胞を特異的な標的細胞として抗ＨＩＶ配列を導入することが可能となる。

ところで、抗ＨＩＶ配列がその効果を発揮するためには、ＨＩＶ遺伝子に相補的に結合することが必須である。ところが、ＨＩＶゲノムは逆転写酵素の読みのあまさから複製ごとに変異を起こしやすく、あらかじめ相補的に結合するようにデザインした抗ＨＩＶ配列が、実際には標的遺伝子と結合できないことが多い。そこで、変異の起こりにくい部位を標的とした抗ＨＩＶ配列の設計が重要となる。ＨＩＶゲノムのｔａｔ部位は比較的変異が起こりにくいうえ、その発現産物であるＴａｔがＨＩＶの複製に必須の調節因子であることから、アンチセンスやリボザイム等の抗ＨＩＶ配列の標的として最適であると考えられている。また、

Ｔａｔが結合するＴＡＲ配列部位も同様の理由でデコイの作用部位としてその効果が期待されている。

しかしながら、これまでに提案されているＨＩＶベクターは、そのゲノム両端のＬＴＲ配列が野生型と同一のものであり、このＬＴＲ配列が導入遺伝子（抗ＨＩＶ配列）のプロモーターとして機能するためには、Ｔａｔによるその活性が必要となる。従って、組換えヒト免疫不全ウイルスベクターを調製する際に抗ＨＩＶ配列によってｔａｔ部位の発現を抑制したり、あるいはその発現産物Ｔａｔを捕捉したりすると、ＬＴＲが不活性化してウイルスベクターの力価を著しく低下させてしまうという問題が存在した。

発明の開示

この発明は、従来のＨＩＶベクターの欠点を解消し、アンチセンス、リボザイム、ＴＡＲデコイ等の抗ＨＩＶ配列がＨＩＶ感染細胞で確実に発現する新しい組換えＨＩＶベクターを提供することを目的としている。

この発明は、ゲノム５′側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されている組換えＨＩＶベクターを提供する。

また、この発明は、少なくともゲノム５′側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されているＨＩＶゲノムを保持するプラスミドベクターと、少なくともＨＩＶゲノムを保持するヘルパープラスミドベクターとを動物細胞にトランスフェクション

し、その培養液からＨＩＶ粒子を採取することを特徴とする組換えＨＩＶの製造方法をも提供する。

なお、この発明においては、組換えＨＩＶベクターが、ＨＩＶの増殖および／または遺伝子発現に対して拮抗的に作用する外来性ＤＮＡ配列を保持すること、さらには、その外来性ＤＮＡ配列が、ＨＩＶゲノムのｔａｔ領域に対するアンチセンス鎖をコードするＤＮＡ配列、ｔａｔ領域に対するリボザイムをコードするＤＮＡ配列、またはＴＡＲ領域に対するデコイをコードするＤＮＡ配列であることを好ましい態様としてもいる。

この発明の組換えＨＩＶベクターは、アンチセンス、リボザイム、ＴＡＲデコイ等の抗ＨＩＶ配列をＨＩＶ感染細胞で確実に発現するため、ＨＩＶ感染症に対する in vivo 遺伝子治療の材料として有用である。

図面の簡単な説明

図１は、この発明の製造方法に用いる組換えプラスミドＣＨＸＮの構成図である。

図２は、この発明の製造方法に用いる組換えプラスミドを導入した細胞が発現したＲＮＡに対するノーザンブロッティングの結果を示す。

図３は、比較例として作成した組換えプラスミドを導入した細胞が発現したＲＮＡに対するノーザンブロッティングの結果を示す。

図４は、この発明の製造方法により得たウイルス粒子と

比較例それぞれの薬剤耐性コロニー数を示す。

発明を実施するための最良の形態

この発明の組換えＨＩＶベクターは、そのゲノム５′側のＬＴＲ配列が、Ｕ３領域をサイトメガロウイルス（以下、ＣＭＶと略記する）のプロモーター配列に置換したハイブリッド型ＬＴＲ（以下、ＣＭＶ－ＨＩＶ・ＬＴＲと略記する）で構成されている。

このような組換えウイルスは、例えば次のような手順で製造することができる。すなわち、少なくとも５′側にＣＭＶ－ＨＩＶ・ＬＴＲを保持する組換えプラスミドベクター（例えば、図１参照）と、少なくともＨＩＶゲノムを保持する組換えプラスミドベクター（ヘルパープラスミド）を各々公知の遺伝子操作法により構築し、これらを公知の方法（例えば、リン酸カルシウム法等）により動物細胞にトランスフェクションする。そしてこのトランスフェクタントを公知の方法で培養し、培養上清中のウイルス粒子を採取することによって、目的とする組換えＨＩＶベクターを得ることができる。また、アンチセンス鎖やリボザイム、デコイ等の抗ＨＩＶ配列を保持する組換え体を作成する場合には、例えば図１に示したようなＣＭＶ－ＨＩＶ・ＬＴＲを保持するプラスミドベクターに、抗ＨＩＶ配列をコードするＤＮＡ配列を挿入結合すればよい。このようにして挿入された抗ＨＩＶ配列は、上記ウイルス粒子に保持され、ｔａｔ領域に対するアンチセンス鎖やリボザイム、あるい

はＴＡＲデコイ等を安定的に発現して、ＨＩＶの増殖や遺伝子発現を効果的に抑制する。

実施例

以下、実施例を示してこの発明をさらに詳しく説明するが、この発明は以下の例に限定されるものではない。

実施例１

（プラスミドの構築）

全てのプラスミドの構築に関する操作は一般的な遺伝子操作法を用いて行なった。組換えプラスミドＣＨＸＮは、図１に示すように５′から３′方向に順次、ＣＭＶ－ＨＩＶ・ＬＴＲ、ネオマイシン耐性遺伝子、およびＨＩＶ・ＬＴＲを有し、これらをＳＶ４０の複製開始点を含むプラスミドベクターに挿入することにより構築した。

実施例２

（組換えプラスミドによる細胞のトランスフェクションと、トランスフェクションされた細胞の評価）

トランスフェクションの方法は一般的なリン酸カルシウム法で行なった。実施例１によって得たＣＨＸＮ　１０μｇと、Ｔａｔコード配列を有するプラスミド（ＴＡＴ／ＬＨまたはＣＧＰＥ）またはＴａｔコード配列を保持しないプラスミド（ＬＨ／Ｂ）とを混合し、滅菌精製水および塩化カルシウム水溶液を添加して全量を０．５ｍｌにした。この混合液を０．５ｍｌのＨＢＳＰ緩衝液中に振とうしながら滴下し、３０分間室温で放置してプラスミドーリン酸

カルシウム共沈物を得た。比較例として5′から3′方向に順次、ＨＩＶ・ＬＴＲ、ヘルペスウイルス由来チミジンキナーゼのプロモーター、ネオマイシン耐性遺伝子、およびＨＩＶ・ＬＴＲを有し、これらをＳＶ４０の複製開始点を含むプラスミドに挿入することにより構築したプラスミドＨＸＮに関しても同様の方法により共沈物を調製した。９ｃｍのディッシュで約７０％コンフレントの状態に培養されたＣｏｓ細胞の培養液中に各共沈物を添加してCO_2インキュベーター内で4時間インキュベーション後、新鮮な培養液に置換してさらに2日間インキュベーションした。

以上のトランスフェクションで細胞内に導入された遺伝子の発現性は、これらの細胞のＲＮＡを塩化セシウムを用いた密度勾配遠心法により抽出し、ノーザンブロッティング法により解析することで評価した。得られた結果を図２および図３に示す。

比較例（プラスミドＨＸＮ）ではＲＮＡの発現がＴａｔ依存的に見られるのに対し（図３）、ＣＨＸＮはＴａｔのあるなしに拘らずＲＮＡの発現が観察された（図２）。

実施例３

（組換えウイルスの調製及び細胞のトランスダクション）

実施例２と同様の方法により、ＣＨＸＮあるいはＨＸＮをＣＧＰＥと共にトランスフェクションした。トランスフェクションの２日後、培養上清を採取しこれをウイルス液とした。ウイルス液を採取する前日に６５ｍｍのディッシュ当たり2×10^5個のＣＤ４陽性Ｈｅｌａ細胞を播種し

ておき、これらのウイルス液またはコントロール群としてトランスフェクションを行なわなかった細胞の培養上清をそれぞれ0．5mlまたは3ml、30μgのポリブレンと共に添加した（トランスダクション）。CO_2インキュベーター内に2日間放置後、トリプシンーEDTA混液により細胞をディッシュから剥がし、85mmのディッシュに再播種した。6時間CO_2インキュベーター内でインキュベーション後、培養液中にG－418が750μg／mlとなるように添加した。さらに7日間インキュベーションし、薬剤耐性を獲得したコロニー数をカウントした。

結果を図4に示す。ウイルスによる遺伝子導入を受けていないコントロール群はコロニーが認められなかった。一方、CHXN群はHXN群と同等かそれ以上の数のコロニーが観察された。この結果は、CHXN、CGPEのトランスフェクションにより組換えウイルスが調製できること、このウイルスは標的細胞に対して遺伝子導入能力があること、および細胞に導入された組換え遺伝子が効率良く発現していることを示している。

産業上の利用可能性

この発明のウイルスベクターは、HIV感染症に対するin vivo遺伝子治療に利用できる。

請求の範囲

1. ゲノム5'側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されている組換えヒト免疫不全ウイルスベクター。

2. ヒト免疫不全ウイルスの増殖および／または遺伝子発現に対して拮抗的に作用する外来性ＤＮＡ配列を保持する請求項１の組換えヒト免疫不全ウイルスベクター。

3. 外来性ＤＮＡ配列が、ヒト免疫不全ウイルス・ゲノムのｔａｔ領域に対するアンチセンス鎖をコードするＤＮＡ配列である請求項２の組換えヒト免疫不全ウイルスベクター。

4. 外来性ＤＮＡ配列が、ヒト免疫不全ウイルス・ゲノムのｔａ領域に対するリボザイムをコードするＤＮＡ配列である請求項２の組換えヒト免疫不全ウイルスベクター。

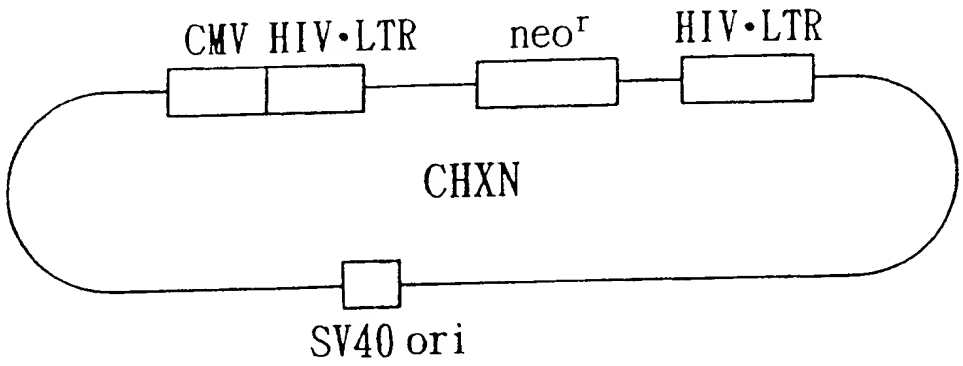
5. 外来性ＤＮＡ配列が、ヒト免疫不全ウイルス・ゲノムのＴＡＲ配列のデコイをコードするＤＮＡ配列である請求項２の組換えヒト免疫不全ウイルスベクター。

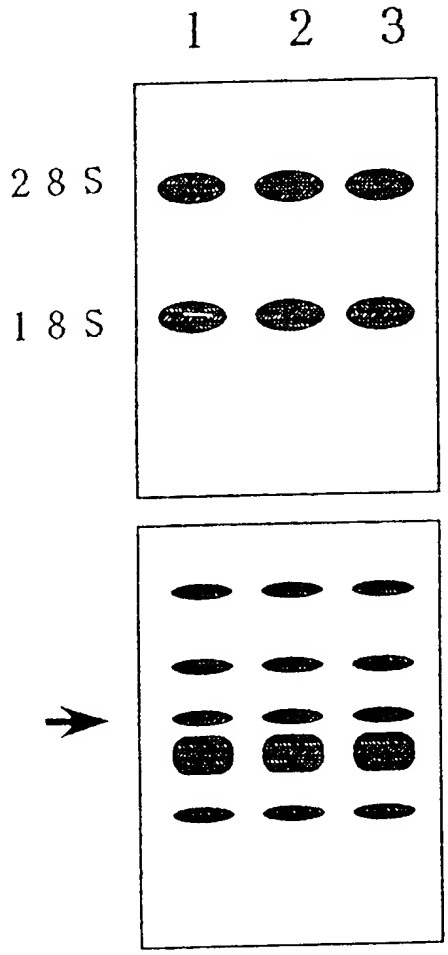
6. 少なくともゲノム5'側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されているヒト免疫不全ウイルスゲノムを保持するプラスミドベクターと、少なくともヒト免疫不全ウイルスゲノムを保持するヘルパープラスミドベクターとを動物細胞にトランスフェクションし、その培養液からヒト免疫不全ウイルス粒子を採取することを特徴とする組換えヒト免疫不全ウ

イルスの製造方法。

7. ゲノム5'側のＬＴＲ配列のＵ３領域がサイトメガロウイルスのプロモーター配列により置換されているヒト免疫不全ウイルスゲノムを保持するプラスミドベクターが、ヒト免疫不全ウイルスの増殖および／または遺伝子発現に対して拮抗的に作用する外来性ＤＮＡ配列をも保持する請求項6のヒト免疫不全ウイルスの製造方法。

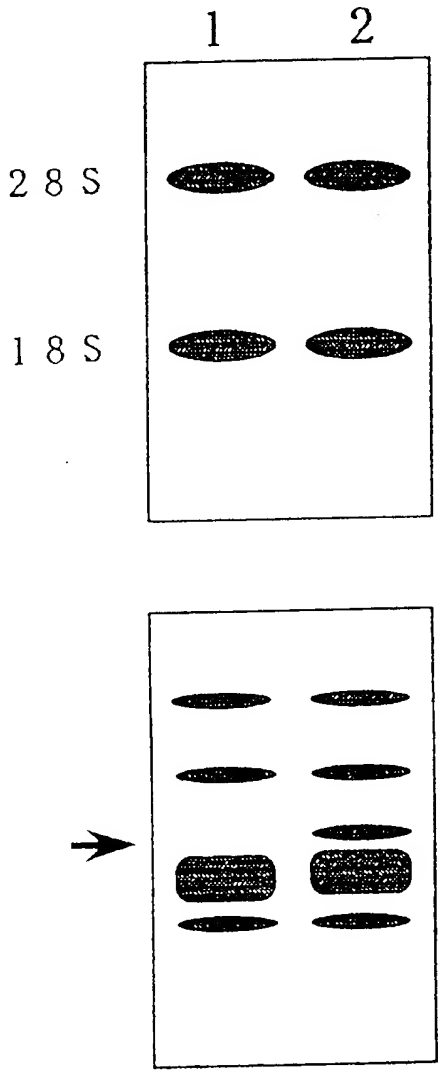
8. 外来性ＤＮＡ配列が、ヒト免疫不全ウイルス・ゲノムのｔａｔ領域に対するアンチセンス鎖、リボザイムまたはＴＡＲ配列に対するデコイをコードするＤＮＡ配列である請求項7の組換えヒト免疫不全ウイルスベクターの製造方法。


CMV HIV·LTR
neo^r
HIV·LTR
CHXN
SV40 ori



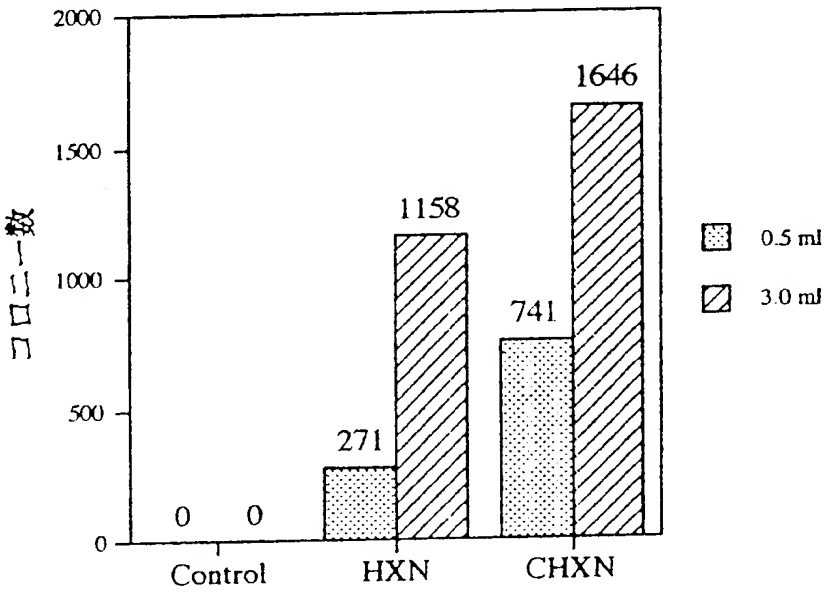
1. CHXN + TAT/LH
2. CHXN +LH/B
3. CHXN + CGPE

2 図



1. HXN + LH/B
2. HXN + TAT/LH

3 図


2000
1500
1000
500
0
コロニー数
1646
1158
741
271
0
0
Control
HXN
CHXN
0.5 ml
3.0 ml

4 図

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/00893

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 C12N15/49, 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^6 C12N15/48-49, 15/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI/L, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Biological Abstracts Vol. 95, No. 73333 & J, Virol., Vol. 67, No. 2 (1993) p.743-752	1, 6 2-5, 7-8
Y	Science, Vol. 258 (1992)p.1485-1488	2, 3, 7, 8
Y	Cell, Vol. 63 (1990) p.601-608	2, 5, 7, 8
Y	Proc. Natl. Acad. Sci. USA, Vol. 89 (1992) p.10802-10806	2, 4, 7, 8

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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July 28, 1995 (28. 07. 95)	August 22, 1995 (22. 08. 95)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

国際調査報告

国際出願番号 PCT/JP 95/00893

A. 発明の属する分野の分類（国際特許分類（IPC））

Int. Cl[6] C12N15/49, 15/86

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int. Cl[6] C12N15/48−49, 15/86

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

WPI/L, BIOSIS

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
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Y	Science, Vol. 258 (1992) p. 1485−1488	2, 3, 7, 8
Y	Cell, Vol. 63 (1990) p. 601−608	2, 5, 7, 8
Y	Proc. Natl. Acad. Sci. USA, Vol. 89 (1992) p. 10802−10806	2, 4, 7, 8

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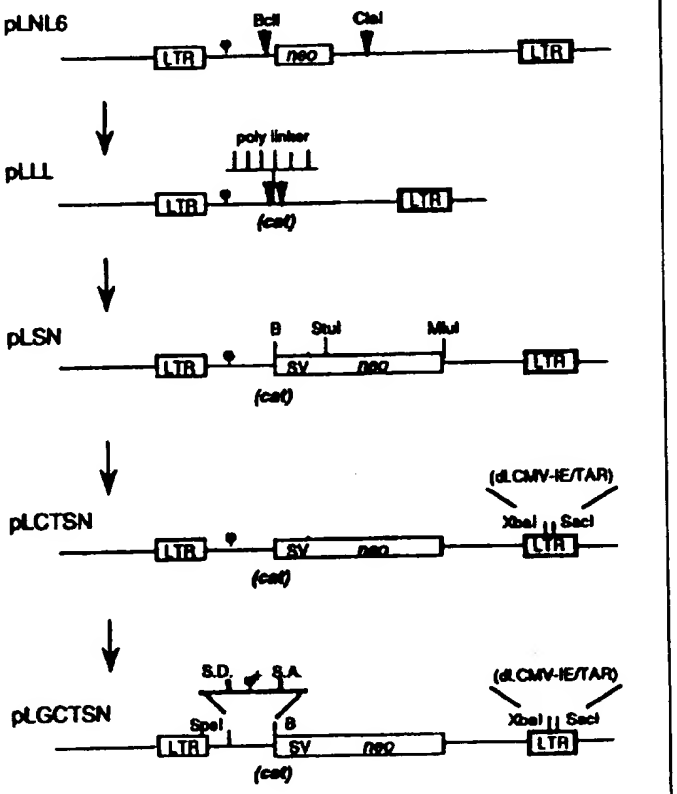
(54) Title: RETROVIRAL VECTORS CONTAINING RECOMBINANT CMV-IE/HIV-TAR/MOLONEY MURINE LEUKEMIA VIRUS LONG TERMINAL REPEATS

(57) Abstract

Novel retroviral vectors were constructed by modifying the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR). A portion of the U3 region of the MoMLV LTR was replaced with the human cytomegalovirus immediate-early enhancer/promoter (CMV-IE) together with the human immunodeficiency virus type 1 (HIV-1) transactivation response element (TAR). Transfection studies involving the hybrid CMV-IE/HIV-1-TAR MoMLV LTR enhancer/promoter demonstrated that this regulatory element increases basal transcriptional levels 10- to 50-fold. Expression from the recombinant MoMLV LTR was further increased by the addition of HIV-1 Tat. Additional vector modifications included the addition of an HIV-1 extended packaging signal and 3' splice acceptor site. Modified retroviral vectors containing the hybrid LTR should be useful for the production of high levels of retroviral and cellular expression products.



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"RETROVIRAL VECTORS CONTAINING RECOMBINANT CMV-IE/HIV-TAR/MOLONEY MURINE LEUKEMIA VIRUS LONG TERMINAL REPEATS"

FIELD OF THE INVENTION

The present invention relates to improved viral vectors useful for the expression of genes at high levels in human cells. These vectors also find use in anti-viral, anti-tumor and/or gene therapy. The improved vectors contain novel long terminal repeats which provide efficient promoters which function in a wide variety of human cell types. The improved vectors also contain additional packaging sequences which results in increased efficiency of packaging the recombinant viral genome. The improved vectors may also contain packaging sequences derived from the HIV.

BACKGROUND OF THE INVENTION

Viral vectors have been used as vehicles for the transfer of genes into many different cell types including whole embryos, fertilized eggs, isolated tissue samples and cultured cell lines. The ability to introduce and express a foreign gene in a cell is useful for the study of gene expression and the elucidation of cell lineages [Watson, J.D., *et al.* (1992) *Recombinant DNA*, Second Edition, W.H Freeman and Co., NY, pp. 256-263]. Retroviral vectors, which integrate into the cellular chromosome, have also been used for the identification of developmentally important genes via insertional mutagenesis [Watson, J.D., *et al.* (1992), *supra*, p.261]. Viral vectors, in particular, retroviral vectors are also used in therapeutic applications (*e.g.*, gene therapy), in which a gene (or genes) is added to a cell to replace a missing or defective gene or to inactivate a pathogen such as a virus.

1. Gene Therapy Strategies For AIDS

Gene therapy has also been proposed for the treatment of chronic infectious diseases such as acquired immunodeficiency syndrome (AIDS). Infection with the human immunodeficiency virus (HIV), a retrovirus, almost always leads to the development of AIDS in humans. Conventional therapeutic treatments, including anti-viral drugs such as reverse transcriptase inhibitors and soluble CD4 have failed to produce a cure for AIDS.

In the absence of any effective long-term therapeutic compounds, alternate strategies for anti-HIV therapy are needed. Recently suggested gene therapy approaches for the treatment of AIDS include: genetic decoy systems (trans-dominant mutants), soluble CD4 binding, toxin-targeting, anti-HIV ribozymes, antisense oligonucleotides, antisense RNA, anti-HIV

intracellular antibodies, anti-HIV antigen-specific T cell transfer, etc. [For review see, Yu, M., Poeschla, E. and Wong-Staal, F. (1994) Gene Ther. 1:13)].

Antisense RNA inhibits gene expression by complementary binding to the target RNA. Antisense RNA transcripts have been used to inhibit replication of retroviruses. For example, antisense RNA was shown to inhibit replication of Rous sarcoma virus in transfected quail cells [Chang, L.-J. and Stoltzfus, C.M. (1985) Mol. Cell. Biol. 5:2341 and Chang, L.-J. and Stoltzfus, C.M. (1987) J. Virol. 61:921]. Since these initial studies, antisense regulation was applied extensively in basic and clinical studies. For example, the anti-HIV antisense oligonucleotide GEM91 is currently being tested in clinical trials in France and the U.S. [reported at the Xth International AIDS Meeting, Yokohama, Japan, Aug. 7-11, 1994].

A drawback to the use of antisense transcripts for therapeutic treatment of retroviral-induced disease is the need to produce large amounts of the antisense transcript in the infected cell. The recent development of trans-cleaving RNA enzymes (ribozymes) has perhaps superseded the antisense strategy by overcoming the stoichiometric limit of the antisense molecules involving RNA-RNA hybrids [Cech, T.R. and Bass, B. (1986) Ann. Rev. Biochem. 55:599]. A ribozyme of the "hammerhead" motif has been shown to act as an anti-HIV agent [Sarver, N. *et al.*, (1990) Science 247:1222]. Human cells stably expressing a hammerhead ribozyme which specifically cleaves the HIV-1 *gag* transcript were shown to substantially reduce the amount of *gag* RNA produced upon infection of these cells with HIV-1. These results suggest that the use of hammerhead ribozymes specific for HIV transcripts may be an effective therapeutic agent provided an efficient means of delivering genes encoding the ribozyme to the cells of an infected individual is developed.

Recently, a ribozyme of the "hairpin" motif has been shown to act as an anti-HIV agent and has gained approval from the NIH Recombinant DNA Advisory Committee as a clinical protocol [Appendices, Human Gene Therapy (1994) 5:147]. In addition to the ribozyme approach, a handful of other genetic approaches including the use of retroviral vectors expressing gp160 [Yu, M., Poeschla, E. and Wong-Staal, F. (1994) Gene Ther. 1:13] or a trans-dominant HIV mutant Rev M10 [Bahner, I. *et al.* (1993) J. Virol. 67:3199 and Appendices, Human Gene Therapy (1994) 5: 79], and adoptive transfer of gene-marked $CD8^+$ T cell clones into patients [Riddell, S.R., Human Gene Therapy (1994) 5:141] have also gained approval for clinical trials.

2. Gene Therapy Strategies For Inborn Errors Of Metabolism

In a few cases, gene therapy has been used to successfully correct inborn errors of metabolism using existing vector systems. For example, the adenosine deaminase gene has been introduced into peripheral blood lymphocytes and cord blood stem cells via retroviral vectors in order to treat patients with severe combined immunodeficiency due to a lack of functional adenosine deaminase [Culver, K.W. *et al.*, (1991) Human Gene Therapy 2:107]. Partial correction of familial hypercholesterolemia has been achieved using existing retroviral vectors to transfer the receptor for low density lipoproteins (LDL) into hepatocytes. However, it was estimated that only 5% of the liver cells exposed to the recombinant virus incorporated the LDL receptor gene with the vector utilized [Grossman, M. *et al.*, (1994) Nat. Genet. 6:335].

A number of single-gene disorders have been targeted for correction using gene therapy. These disorders include hemophilia (lack of Factor VIII or Factor IX), cystic fibrosis (lack of cystic fibrosis transmembrane regulator), emphysema (defective α-1-antitrypsin), thalassemia and sickle cell anemia (defective synthesis of β-globin), phenylketonuria (deficient phenylalanine hydroxylase) and muscular dystrophy (defective dystrophin) [See for review, Miller, A.D. (1992) Nature 357:455]. Human gene transfer trials have been approved for a number of these diseases.

3. Gene Therapy Strategies For Cancer

In addition to replacement of defective genes, it has been proposed that viral vectors could be used to deliver genes designed to stimulate immunity against or to otherwise destroy tumor cells. Retroviral vectors containing genes encoding tumor necrosis factor (TNF) or interleukin-2 (IL-2) have been transferred into tumor-infiltrating lymphocytes in patients [Kasid, A. *et al.* Proc. Natl. Acad. Sci. (1990) and Rosenberg, S. A. Human Gene Therapy 5: 140 (1994)]. It is postulated that the secretion of TNF or IL-2 will stimulate a tumor-specific immune response resulting in the destruction of the tumor or the recruitment of effective tumor infiltrating lymphocytes from nearby lymph nodes. Other proposed anti-tumor gene therapy strategies include the delivery of toxin genes to the tumor cell.

Applications of antisense genes or antisense oligonucleotides in inhibition of oncogenes and modulation of growth factors have the potential to reduce the mortality of cancer, in particular, human leukemia [For review see, Gewirtz A.M. (1993) Stem Cells 3:96 and Neckers, L. and Whitesell, L. (1993) Am. J. of Physiol. 265:L1].

4. Current Viral Vector Systems

In view of the wide variety of potential anti-HIV genes available for therapy, it is clear that an efficient means of delivering these genes is sorely needed in order to fulfill the promise of gene therapy as a means of treating HIV infection. Several viral systems including murine retrovirus, adenovirus, parvovirus (adeno-associated virus), vaccinia virus, and herpes virus have been developed as therapeutic gene transfer vectors [For review see, Nienhuis, A.W. *et al.* (1993) Hematology, Vol. 16:*Viruses and Bone Marrow*, Young, N.S. ed. Chapter 12, pp. 353-414]. Viral vectors provide a more efficient means of transferring genes into cells as compared to other techniques such as calcium phosphate or DEAE-dextran-mediated transfection, electroporation or microinjection. It is believed that the efficiency of viral transfer is due to the fact that the transfer of DNA is a receptor-mediated process (*i.e.*, the virus binds to a specific receptor protein on the surface of the cell to be infected).

While many viral vector systems are available, virtually all of the current human gene therapy trials use retroviral vectors derived from the amphotropic Moloney murine leukemia virus (M-MuLV) for gene transfer [Miller, A.D. and Buttimore, C. (1986) Mol. Cell. Biol. 6:2895]. The M-MuLV system has several advantages: 1) this specific retrovirus can infect many different cell types, 2) established packaging cell lines are available for the production of recombinant M-MuLV viral particles and 3) the transferred genes are permanently integrated into the target cell chromosome. The established M-MuLV vector systems comprise a DNA vector containing a small portion of the retroviral sequence (the viral long terminal repeat or "LTR" and the packaging or "psi" signal) and a packaging cell line. The gene to be transferred is inserted into the DNA vector. The viral sequences present on the DNA vector provide the signals necessary for the insertion or packaging of the vector RNA into the viral particle and for the expression of the inserted gene. The packaging cell line provides the viral proteins required for particle assembly [Markowitz, D. *et al.* (1988) J. Virol. 62:1120].

The vector DNA is introduced into the packaging cell by any of a variety of techniques (*e.g.*, calcium phosphate coprecipitation, electroporation, etc.). The viral proteins produced by the packaging cell mediate the insertion of the vector sequences in the form of RNA into viral particles which are shed into the culture supernatant. The M-MuLV system has been designed to prevent the production of replication-competent virus as a safety measure. The recombinant viral particles produced in these systems can infect and integrate into the target cell but cannot spread to other cells. These safeguards are necessary to prevent the spread of

the recombinant virus from the treated patient and to avoid the possibility of helper virus-induced disease [Miller, A.D. and Buttimore, C. (1986) Mol. Cell. Biol. 6:2895 and Markowitz, D. *et al.*, *supra*].

Despite these advantages, existing retroviral vectors are limited by several intrinsic problems: 1) they do not infect non-dividing cells [Miller, D.G., *et al.*, (1990) Mol. Cell. Biol. 10:4239], 2) they produce only low titers of the recombinant virus [Miller, A. D. and Rosman G. J. (1989) BioTechniques 7: 980 and Miller, A. D. (1992) Nature 357: 455], and 3) they express foreign proteins at low levels and often get "turned-off" or inactivated after integration [Miller, A. D. (1992) Nature 357: 455]. The low production of recombinant virus produced by the M-MuLV system (*e.g.*, 10^6/ml) compared to the adenoviral system (up to 10^{12}/ml) means that human cells are infected at a very low efficiency. This low efficiency is particularly problematic when the target cell type is represented at very low numbers in the tissue to be infected. Although the hematopoietic stem cell is a preferred target for gene therapy in a large number of disorders, these cells are present at very low frequencies. For example, totipotent embryonic stem cells have been reported to occur at a frequency of 10^{-4} to 10^{-5} in bone marrow [B.R. Glick and J.J. Pasternak, *Molecular Biotechnology*, American Society for Microbiology, 1994, p. 412]. Thus, the low titer produced by existing M-MuLV vector systems is problematic for stem cell infection.

In addition, the promoter present in the M-MuLV LTR is quite weak compared with other viral promoters such as the human cytomegalovirus immediate early (CMV-IE) enhancer/promoter. In order to increase expression of the genes carried on the retroviral vector, internal promoters possessing stronger activities than the M-MuLV promoter have been utilized. However, the inclusion of an internal promoter to drive the expression of the inserted gene does not always lead to increased levels of expression [Robinson, D., Elliott, J. F. and Chang, L.-J. (1995) Gene Therapy 2:269]. Apparently, the activity of the internal promoter is significantly decreased because of interference from the upstream M-MuLV promoter (*i.e.*, transcriptional read-through interference). The dual transcription-unit construct is, however, a common feature in almost all M-MuLV vectors. Given these limitations, it is clear that improved vector systems are urgently needed to provide a means of delivering and expressing genes efficiently in mammalian cells, particularly human cells. Improved vectors will aid the study of gene expression and development and are necessary if the promise of gene therapy is to be realized.

SUMMARY OF THE INVENTION

The present invention contemplates improved viral vectors useful for the expression of genes at high levels in human cells. These vectors also find use in anti-viral, anti-tumor and/or gene therapy. The improved vectors contain novel long terminal repeats which provide efficient promoters which function in a wide variety of human cell types. The improved vectors also contain additional packaging sequences which result in increased efficiency of packaging the recombinant viral genome.

In one embodiment, the invention comprises a recombinant Moloney murine leukemia virus long terminal repeat which is activated by the human immuno-deficiency virus 1 Tat protein, wherein the recombinant long terminal repeat has increased promoter activity relative to the wild type Moloney murine leukemia virus long terminal repeat in human cells. This increased activity can be readily assayed in a side-by-side comparison with both the recombinant LTR and the wild type LTR in a vector in host cells (*e.g.*, human). In a preferred embodiment, the recombinant long terminal repeat contains the human cytomegalovirus immediate early enhancer/promoter and the HIV-1 TATA and TAR elements in place of the Moloney murine leukemia virus promoter element in the U3 region of the long terminal repeat. An example of such a construct is given having the sequence shown in SEQ ID NO:17.

In one embodiment, the recombinant long terminal repeat of this invention is contained on a recombinant murine amphotropic retroviral vector. This retroviral vector comprises in operable order: a) a first long terminal repeat; b) a packaging signal joined to this first long terminal repeat; and c) a second long terminal repeat joined to the packaging signal. In a preferred embodiment, the vector further comprises an oligonucleotide having a nucleotide sequence encoding a selectable marker gene, wherein the selectable marker gene is operably linked between the packaging signal and second long terminal repeat. It is contemplated that the selectable marker gene be a dominant selectable marker gene. In a preferred embodiment, the dominant selectable marker gene is the neomycin phosphoribosyltransferase gene. An example of such a preferred embodiment is given by the vector pLCTSN (deposited with the American Type Culture Collection).

In another embodiment, the recombinant long terminal repeat of the present invention includes a packaging signal comprising an extended Moloney murine leukemia virus packaging signal, wherein this extended packaging signal results in an increased packaging efficiency of the recombinant vector. An example of such a preferred embodiment is given

by the vector pLGCTSN (deposited with the American Type Culture Collection). In an alternative preferred embodiment, the packaging signal comprises a human immunodeficiency virus packaging signal. It is contemplated that this human immunodeficiency virus packaging signal consist of the sequence listed in SEQ ID NO:10 or the sequence listed in SEQ ID NO:11.

In an alternative embodiment of the present invention, the recombinant murine amphotropic retroviral vector contains the following elements in operable order: a) a first long terminal repeat; b) a packaging signal joined to this first long terminal repeat; c) a polylinker joined to the packaging signal; and d) a second long terminal repeat consisting of the sequence listed in SEQ ID NO:17 joined to the polylinker.

In a further embodiment, the recombinant vector further comprises a selectable marker inserted into the polylinker. In a preferred embodiment, the selectable marker is a dominant selectable marker. It is contemplated that the dominant selectable marker is the neomycin phosphoribosyltransferase gene.

In one embodiment, the recombinant vector contains a packaging signal comprising an extended Moloney murine leukemia virus packaging signal. In a preferred embodiment, this packaging signal comprises a packaging signal derived from human immunodeficiency virus I (HIV-1). It is contemplated that the HIV-1 packaging signal consist of SEQ ID NO:10 or SEQ ID NO:11.

The present invention contemplates improving existing vectors. For example, the clinically approved retroviral vector pLNL6 was modified to generate pLLL (deposited with the ATCC). The present invention contemplates modification of the pLLL vector to generate other preferred vectors.

As noted above, the present invention contemplates that the improved viral vectors can be used for the expression of genes at high levels in human cells. Specifically, the present invention contemplates a method for expressing a gene in a human cell line, comprising the steps of a) providing a human cell line, and a retroviral vector containing the recombinant long terminal repeat of SEQ ID NO:17 and a gene of interest; and b) introducing the retroviral vector into the human cell line under conditions which allow the expression of the gene of interest.

In one embodiment of this method, the vector further contains a selectable marker. In a preferred embodiment, the selectable marker is a dominant selectable marker. It is

contemplated that the dominant selectable marker is the neomycin phosphoribosyltransferase gene.

An alternative embodiment of this method comprises the further step of: c) exposing the human cell line to conditions, wherein the conditions allow only those human cells expressing the selectable marker to grow. In a preferred embodiment, the conditions comprise a selective medium. In a particularly preferred embodiment, the selective medium contains the antibiotic G418.

DESCRIPTION OF THE FIGURES

Figure 1 is a simplified schematic illustration showing the production of packaged retrovirus vector RNA in a packaging cell line.

Figure 2 shows an autoradiograph of a chloramphenicol acetyltransferase (CAT) assay showing the relative promoter strengths of HIV, CMV-IE and MLV in HeLa cells.

Figure 3 shows the map of the retroviral vector pLLL. Selected restriction enzyme sites are indicated.

Figure 4 shows the map of the retroviral vector pLLLSV40. Selected restriction enzyme sites are indicated.

Figure 5 shows the map of the retroviral vector pLLLSVtat. Selected restriction enzyme sites are indicated.

Figure 6 shows the map of the retroviral vector pLLLgpt. Selected restriction enzyme sites are indicated.

Figure 7 shows the map of the retroviral vector pLLLgptSVtat. Selected restriction enzyme sites are indicated.

Figure 8 is a simple schematic of the organization of the plasmids used in the experiment depicted in Figure 9.

Figure 9 shows an autoradiograph of a CAT assay depicting promoter activities in the presence of Tat. Percent conversion is shown.

Figure 10 is a schematic showing the pMT-cat and pMCT-cat constructs.

Figure 11 shows an autoradiograph of a CAT assay depicting the basal and Tat-induced promoter activity in AA2 cells.

Figure 12 shows an autoradiograph of a CAT assay depicting the relative levels of CAT expression in CEM-TART cells.

Figure 13 shows an autoradiograph of a Northern blot depicting the results of a trans-activation initiation experiment.

Figure 14 shows an autoradiograph of a RNA protection assay depicting the results of a trans-activation initiation experiment.

Figure 15 schematically depicts the probe used in the experiment shown in Figure 14.

Figure 16 is a schematic of the modifications made to produce the modified MuLV vectors.

Figure 17 shows an autoradiograph of a CAT assay depicting the long term expression of genes in the pLSN-cat and pLCTSN-cat constructs in HeLa cells.

Figure 18 shows an autoradiograph of a CAT assay depicting the long term expression of genes in the pLSN-cat and pLCTSN-cat constructs in transduced HepG2 cells in the presence or absence of Tat.

Figure 19 shows the map of the retroviral vector pLLL-PAK100. Selected restriction enzyme sites are indicated.

Figure 20 shows the map of the retroviral vector pLLLgpt-PAK100. Selected restriction enzyme sites are indicated.

Figure 21 shows the map of the retroviral vector pLLLgpt-PAK140. Selected restriction enzyme sites are indicated.

Figure 22 shows the map of the retroviral vector pLLLSVhyg. Selected restriction enzyme sites are indicated.

Figure 23 shows the map of the retroviral vector pLCTSN-PAK. Selected restriction enzyme sites are indicated.

Figure 24 shows the reverse transcriptase dot blot results of PA317 and GP-AM12 culture supernatants.

Figure 25 provides schematic diagrams of the pLSNnef. pLSN-PAK100. pLCTSN-PAK140 and pLGCTSN-PAK140 retroviral vectors.

Figure 26A shows RT actitivity present in culture supernatants from H9 cells transduced with various retroviral vectors and then infected with HIV-1_{HL4-3} at a MOI of 0.001 versus days post-infection.

Figure 26B shows RT actitivity present in culture supernatants from AA2 cells were transduced with various retroviral vectors and then infected with HIV-1_{HL4-3} at a MOI of 0.001 versus days post-infection.

Figure 27A shows RT actitivity present in culture supernatants from H9 cells were transduced with various retroviral vectors and then infected with HIV-1_{IIIB} at a MOI of 0.2 versus days post-infection.

Figure 27B shows RT actitivity present in culture supernatants from H9 cells were transduced with various retroviral vectors and then infected with HIV-2-ROD at a MOI of 0.2 versus days post-infection.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "antisense" is used in reference to RNA sequences which are complementary to a specific RNA sequence (*e.g.*, mRNA). Included within this definition are antisense RNA ("asRNA") molecules involved in gene regulation by bacteria. Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced into an embryo, this transcribed strand combines with natural mRNA produced by the embryo to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand.

As used herein, the term "polyA$^+$ RNA" refers to RNA molecules having a stretch of adenine nucleotides at the 3' end. this polyadenine stretch is also referred to as a "poly-A tail". Eucaryotic mRNA molecules contain poly-A tails and are referred to as polyA$^+$ RNA.

As used herein, the term "ribozyme" is used in reference to RNA molecules with catalytic activity. It is intended that this term will encompass any catalytic RNA molecule, including, but not limited to, ribonuclease P, and pre-rRNA molecules.

As used herein, the terms "self-trimming" and "self-cleavage" refer to the ability of ribozymes and other molecules to cleave their own structures or sequences.

As used herein, the term "trans" is used in reference to the positioning of genes of interest on the different strands of nucleic acid (*e.g.*, alleles present on the two chromosomes of a chromosomal pair). The term "trans-acting" is used in reference to the controlling effect of a regulatory gene on a gene present on a different chromosome. In contrast to promoters,

repressors are not limited in their binding to the DNA molecule that includes their genetic information. Therefore, repressors are sometimes referred to as trans-acting control elements.

The term "trans-activation" as used herein refers to the activation of gene sequences by factors encoded by a regulatory gene which is not necessarily contiguous with the gene sequences which it binds to and activates. For example, the HIV-1 regulatory protein Tat is encoded by the *tat* gene and binds to and activates (*i.e.*, trans-activates) expression from the HIV LTR.

As used herein, the term "cis" is used in reference to the presence of genes on the same chromosome. The term "cis-acting" is used in reference to the controlling effect of a regulatory gene on a gene present on the same chromosome. For example, promoters, which affect the synthesis of downstream mRNA are cis-acting control elements.

As used herein, the term "retrovirus" is used in reference to RNA viruses which utilize reverse transcriptase during their replication cycle. The retroviral genomic RNA is converted into double-stranded DNA by reverse transcriptase. This double-stranded DNA form of the virus integrates into the chromosome of the infected cell and is referred to as a "provirus." The provirus serves as a template for RNA polymerase II and directs the expression of RNA molecules which encode the structural proteins and enzymes needed to produce new viral particles. At each end of the provirus are structures called "long terminal repeats" or "LTRs". The LTR contains numerous regulatory signals including transcriptional control elements, polyadenylation signals and sequences needed for replication and integration of the viral genome. The viral LTR is divided into three regions called U3, R and U5.

The U3 region contains the enhancer and promoter elements. The U5 region contains the polyadenylation signals. The R (repeat) region separates the U3 and U5 regions and transcribed sequences of the R region appear at both the 5' and 3' ends of the viral RNA

As used herein, the term "endogenous virus" is used in reference to an inactive virus which is integrated into the chromosome of its host cell (often in multiple copies), and can thereby exhibit vertical transmission. Endogenous viruses can spontaneously express themselves and may result in malignancies.

As used herein, the terms "amphotrope" and "amphotropic" are used in reference to endogenous viruses that readily multiply in cells of the species in which they were induced, as well as cells of other species.

As used herein, the term "ecotrope" and "ecotropic" are used in reference to endogenous viruses that multiply readily in cells of the species in which they were induced, but cannot multiply in cells of other species.

As used herein, the term "xenotrope" and "xenotropic" are used in reference to endogenous viruses that cannot infect cells of the species in which they were induced, but can infect and multiply in cells of other species.

As used herein, the term "provirus" is used in reference to a virus that is integrated into a host cell chromosome (or genome), and is transmitted from one cell generation to the next, without causing lysis or destruction of the host cell. The term is also used in reference to a duplex DNA sequence present in an eucaryotic chromosome, which corresponds to the genome of an RNA retrovirus.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*.

The term "T25 flask" refers to a tissue culture flask having a growth surface area of 25 square centimeters.

As used herein, the term "packaging signal" or "packaging sequence" refers to sequences located within the retroviral genome which are required for insertion of the viral RNA into the viral capsid or particle. Several retroviral vectors use the minimal packaging signal (also referred to as the psi sequence) needed for encapsidation of the viral genome. This minimal packaging signal encompasses bases 212 to 563 of the Mo-MuLV genome [Mann *et al.* (1983) Cell 33:153].

As used herein, the term "extended packaging signal" or "extended packaging sequence" refers to the use of sequences around the psi sequence with further extension into the *gag* gene. In Mo-MuLV this extended packaging sequence corresponds to the region encompassing base 1039 to base 1906 [Akagi, T.*et al.* (1991) Gene 106:255]. The frequently used M-MuLV vector, pLNL6 [Bender, M.A., *et al.* (1987) J. Virol. 61:1639], contains the entire 5' region of the genome including an extended packaging signal from bases 206 to 1039 of the Moloney murine sarcoma virus genome [numbering from Supplements and Appendices in *RNA Tumor Viruses*, 2nd Ed. (1985) pp. 986-988]. The inclusion of these additional packaging sequences increases the efficiency of insertion of vector RNA into viral particles

As used herein, the term "packaging cell lines" is used in reference to cell lines that express viral structural proteins (*e.g., gag, pol* and *env*), but do not contain a packaging signal. For example, a cell line has been genetically engineered to carry at one chromosomal site within its genome, a 5'-LTR-*gag-pol*3'-LTR fragment that lacks a functional psi$^-$ sequence (designated as Δpsi), and a 5'-LTR-*env*-3'-LTR fragment which is also Δpsi located at another chromosomal site. While both of these segments are transcribed constitutively, because the psi$^-$ region is missing and the viral RNA molecules produced are less than full-size, empty viral particles are formed.

When retroviral vector DNA is transfected into the cells, it becomes integrated into the chromosomal DNA and is transcribed, thereby producing full-length retroviral vector RNA that has a psi$^+$ sequence. Under these conditions, only the vector RNA is packaged into the viral capsid structures These complete, yet replication-defective, virus particles can then be used to deliver the retroviral vector to target cells with relatively high efficiency.

Figure 1 is a simplified schematic showing the production of packaged retrovirus vector RNA in a packaging cell line. In this figure, the released viral particles carry a remedial gene (Gene X) and a selectable marker gene for resistance to neomycin (*Neo*^{r}).

As used herein, the term "remedial gene" refers to a gene whose expression is desired in a cell to correct an error in cellular metabolism, to inactivate a pathogen or to kill a cancerous cell. For example, the adenosine deaminase (ADA) gene is the remedial gene when carried on a retroviral vector used to correct ADA deficiency in a patient.

As used herein, the term "selectable marker" refers to the use of a gene which encodes an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be "dominant"; a dominant selectable marker encodes an enzymatic activity which can be detected in any eucaryotic cell line. Examples of dominant selectable markers include the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the *neo* gene) which confers resistance to the drug G418 in mammalian cells, the bacterial hygromycin G phosphotransferase (*hyg*) gene which confers resistance to the antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the *gpt* gene) which confers the ability to grow in the presence of mycophenolic acid. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (*tk*) gene which is used in conjunction with *tk*$^-$ cell lines, the CAD gene which is used in conjunction with CAD-

deficient cells and the mammalian hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene which is used in conjunction with *hprt* cell lines. A review of the use of selectable markers in mammalian cell lines is provided in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp.16.9-16.15.

As used herein, the terms "packaging sequence," "packaging signal," and "psi" are used in reference to the non-coding sequence required for encapsidation of retroviral RNA strands during viral particle formation.

As used herein, the term "retroviral vector" is used in reference to retroviruses which have been modified so as to serve as vectors for introduction of nucleic acid into cells.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector."

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in procaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eucaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "genetic cassette" as used herein refers to a fragment or segment of DNA containing a particular grouping of genetic elements. The cassette can be removed and inserted into a vector or plasmid as a single unit.

The term "transfection" as used herein refers to the introduction of foreign DNA into eucaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

As used herein, the term "transduction" refers to the delivery of a gene(s) using a retroviral vector by means of infection rather than by transfection. For example, an anti-HIV gene carried by a retroviral vector can be transduced into a host cell through infection and provirus integration.

As used herein, the term "TATA element" or "TATA box" is used in reference to a segment of DNA, located approximately 19-27 base pairs upstream from the start point of eucaryotic structural genes, to which RNA polymerase binds. The TATA box is approximately 7 base pairs in length, often comprising the sequence "TATAAAA." The TATA box is also sometimes referred to as the "Hogness box."

The term "CAAT box" or "CAAT element" refers to a conserved DNA sequence located approximately 75 bp upstream from the start point of eucaryotic structural genes, to which RNA polymerase binds.

As used herein, the term "*tat*" is used in reference to the HIV gene which encodes "Tat," a protein which induces high-level expression of HIV genes.

As used herein, the term "long terminal repeat (LTR)" is used in reference to domains of base pairs located at the ends of retroviral DNA's. These LTRs may be several hundred base pairs in length. LTR's often provide functions fundamental to the expression of most eucaryotic genes (*e.g.*, promotion, initiation and polyadenylation of transcripts).

As used herein, the term "TAR" is used in reference to the "trans-activation response" genetic element located in the U5 region of the HIV LTR. This element mediates the action of *tat*, by physically binding to the viral trans-activator *tat*.

As used herein, the term "adoptive transfer" is used in reference to the transfer of one function to another cell or organism. For example, in "adoptive immunity," transfer of an immune function is made from one organism to another through the transfer of immunologically competent cells.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between

nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\%\ G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.*, Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* (1985). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of hybridizing to another oligonucleotide of interest. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is further contemplated that the oligonucleotide of interest (*i.e.*, to be detected) will be labelled with a reporter molecule. It is also contemplated that both the probe and oligonucleotide of interest will be labelled. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target" refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195 and 4,683,202, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of

thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid.

For example, in the case of Qβ replicase, MDV-1 RNA is the specific template for the replicase [D.L. Kacian *et al.*, Proc. Nat. Acad. Sci USA 69:3038 (1972)]. Other nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters [M. Chamberlin *et al.*, Nature 228:227 (1970)]. In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides where there is a mismatch between the oligonucleotide substrate and the template at the ligation junction [D.Y. Wu and R. B. Wallace, Genomics 4:560 (1989)]. Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences [*PCR Technology*, H.A. Erlich (ed.) (Stockton Press 1989)].

Some amplification techniques take the approach of amplifying and then detecting target; others detect target and then amplify probe. Regardless of the approach, nucleic acid must be free of inhibitors for amplification to occur at high efficiency.

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the term "nested primers" refers to primers that anneal to the target sequence in an area that is inside the annealing boundaries used to start PCR [K.B. Mullis, *et al.*, Cold Spring Harbor Symposia, Vol. II, pp.263-273 (1986)]. Because the nested primers anneal to the target inside the annealing boundaries of the starting primers, the predominant PCR-amplified product of the starting primers is necessarily a longer sequence, than that defined by the annealing boundaries of the nested primers. The PCR-amplified product of the nested primers is an amplified segment of the target sequence that cannot, therefore, anneal with the starting primers. Advantages to the use of nested primers include the large degree of specificity, as well as the fact that a smaller sample portion may be used and yet obtain specific and efficient amplification.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleoside triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along

with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, the term "an oligonucleotide having a nucleotide sequence encoding a gene" means a DNA sequence comprising the coding region of a gene or in other words the DNA sequence which encodes a gene product. The coding region may be present in either a cDNA or genomic DNA form. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

As used herein. the term "transcription unit" refers to the segment of DNA between the sites of initiation and termination of transcription and the regulatory elements necessary for the efficient initiation and termination. For example. a segment of DNA comprising an enhancer/promoter. a coding region and a termination and polyadenylation sequence comprises a transcription unit.

As used herein. the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example. a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals. polyadenylation signals. termination signals. *etc.* (defined *infra*).

Transcriptional control signals in eucaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription [Maniatis. T. *et al.*. Science 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of eucaryotic sources including genes in yeast. insect and mammalian cells and viruses (analogous control elements. *i.e.*. promoters. are also found in procaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eucaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types [for review see Voss. S.D. *et al.*. Trends Biochem. Sci.. 11:287 (1986) and Maniatis. T. *et al.*. *supra* (1987)]. For example. the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells [Dijkema. R. *et al.*. EMBO J. 4:761 (1985)]. Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1α gene [Uetsuki. T. *et al.*. J. Biol. Chem.. 264:5791 (1989). Kim. D.W. *et al.*. Gene 91:217 (1990) and Mizushima. S. and Nagata. S.. Nuc. Acids. Res.. 18:5322 (1990)] and the long terminal repeats of the Rous sarcoma virus [Gorman. C.M. *et al.*. Proc. Natl. Acad. Sci. USA 79:6777 (1982)] and the human cytomegalovirus [Boshart. M. *et al.*. Cell 41:521 (1985)].

As used herein. the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (*i.e.*. the functions provided by a promoter element and an enhancer element. see above for a discussion of these functions). For example. the long terminal repeats of retroviruses contain

both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter.

The term "factor" refers to a protein or group of proteins necessary for the transcription or replication of a DNA sequence. For example, SV40 T antigen is a replication factor which is necessary for the replication of DNA sequences containing the SV40 origin of replication. Transcription factors are proteins which bind to regulatory elements such as promoters and enhancers and facilitate the initiation of transcription of a gene.

Promoters and enhancers may bind to specific factors which increase the rate of activity from the promoter or enhancer. These factors may be present in all cell types or may be expressed in a tissue-specific manner or in virus infected cells. In the absence of such a factor the promoter may be inactive or may produce a low level of transcriptional activity. Such a low level of activity is referred to as a baseline or "basal" rate of activity. Additionally, viral promoter and enhancers may bind to factors encoded by the virus such that the viral promoter or enhancer is "activated" in the presence of the viral factor (in a virus infected cell or in a cell expressing the viral factor). The level of activity in the presence of the factor (*i.e.*, activity "induced" by the factor) will be higher than the basal rate.

Different promoters may have different levels of basal activity in the same or different cell types. When two different promoters are compared in a given cell type in the absence of any inducing factors, if one promoter expresses at a higher level than the other it is said to have a higher basal activity.

The activity of a promoter and/or enhancer is measured by detecting directly or indirectly the level of transcription from the element(s). Direct detection involves quantitating the level of the RNA transcripts produced from that promoter and/or enhancer. Indirect detection involves quantitation of the level of a protein, often an enzyme, produced from RNA transcribed from the promoter and/or enhancer. A commonly employed assay for promoter or enhancer activity utilizes the chloramphenicol acetyltransferase (CAT) gene. A promoter and/or enhancer is inserted upstream from the coding region for the CAT gene on a plasmid; the plasmid is introduced into a cell line. The levels of CAT enzyme are measured. The level of enzymatic activity is proportional to the amount of CAT RNA transcribed by the

cell line. This CAT assay therefore allows a comparison to be made of the relative strength of different promoters or enhancers in a given cell line. When a promoter is said to express at "high" or "low" levels in a cell line this refers to the level of activity relative to another promoter which is used as a reference or standard of promoter activity.

The improved recombinant vectors of the invention contain recombinant long terminal repeats (LTRs) in which the M-MuLV U3 region is replaced by the CMV-IE enhancer/promoter and the HIV TATA and TAR elements. This novel LTR (SEQ ID NO:17) exhibits increased promoter activity in most human cell lines than does the parental M-MuLV LTR. The recombinant LTR has a higher basal promoter activity in human cells as compared to the parental or wild type M-MuLV LTR. Furthermore, the recombinant LTR is inducible by the Tat protein such that in cells expressing the HIV Tat protein, the activity of the recombinant LTR is enhanced.

When it is said that the recombinant LTR exhibits stronger or increased promoter activity than the parental M-MuLV LTR this means that, relative to the value obtained for the activity of the M-MuLV promoter, the recombinant LTR (present in pMCT: SEQ ID NO:17) exhibits a value at least 2 fold higher (typically 2 to 10 fold higher values obtained when promoter activity is measured by determining the percent conversion of chloramphenicol to acetylated chloramphenicol) than that obtained using the M-MuLV LTR in the HeLa, HepG2 and HuH-7 cell lines. The promoter activity may be measured by placing a reporter gene, such as the CAT gene, under the control of either the M-MuLV LTR or the pMCT LTR. Human cell lines are transfected with the DNAs and CAT activity is assayed. A comparison between the activity levels (judged by the conversion of chloramphenicol to acetylated chloramphenicol) is made by obtaining reproducible mean values from at least three independent transfection experiments; the values are normalized to the expression of an internal control gene to account for difference in uptake of DNA or cell viability between experiments and within an experiment.

The recombinant LTR of SEQ ID NO:17 is further characterized by its ability to be up-regulated in the presence of the HIV Tat protein. The promoter activity of the recombinant LTR is said to be inducible by Tat. This means that the level of activity from the recombinant LTR increases at least 2 fold (typically 2 to 16 fold higher values obtained when promoter activity is measured by determining the percent conversion of chloramphenicol to acetylated chloramphenicol) in the presence of the Tat protein in a human cell line when compared to the activity in the same human cell line in the absence of Tat. The ability of a

promoter to be activated by Tat is measured by placing a reporter gene (*e.g.*, CAT gene) under the direction of the promoter (*i.e.*, downstream of the promoter sequences) and introducing the DNA construct into a human cell in the presence and the absence of a construct directing the expression of the Tat protein. The activity of the reporter gene is measured using a suitable assay (such as the CAT assay). A comparison between the activity levels (judged by the conversion of chloramphenicol to acetylated chloramphenicol when the CAT assay is employed) is made by obtaining reproducible mean values from at least three independent transfection experiments: the values are normalized to the expression of an internal control gene to account for difference in uptake of DNA or cell viability between experiments and within an experiment.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site [Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp. 16.7-16.8]. A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eucaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam* HI/*Bcl* I restriction fragment and directs both termination and polyadenylation [Sambrook, J., *supra*, at 16.6-16.7].

Eucaryotic expression vectors may also contain "viral replicons "or "viral origins of replication." Viral replicons are viral DNA sequences which allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors

which contain either the SV40 or polyoma virus origin of replication replicate to high copy number (up to 10^4 copies/cell) in cells that express the appropriate viral T antigen. Vectors which contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number (~100 copies/cell).

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up foreign DNA but have failed to integrate this DNA.

As used herein, the term "gene of interest" refers to the gene inserted into the polylinker of an expression vector. When the gene of interest encodes a gene which provides a therapeutic function (such as an anti-HIV gene), the gene of interest may be alternatively called a remedial gene.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

The PAK sequences derived from the HIV-1, when present on a retroviral vector in a cell which also expresses the HIV-1 genome, is "capable of inhibiting the insertion" of said HIV-1 genome into HIV-1 particles. This inhibition or interference in the packaging of the HIV-1 genome is detected by a drop in the amount of infectious HIV-1 particles in the cell expressing both the PAK sequences and HIV. The art knows of several ways to measure the titer or number of infectious HIV-1 particles. An inhibition of at least two-fold in the titer of infectious HIV particles is considered significant.

The term "lipofection" refers to a technique for the introduction of nucleic acids into a cell. Lipofection utilizes a liposome formulation of cationic lipids such as N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate

and a neutral lipid such as dioleoyl phosphatidylethanolamine. The liposomes complex with nucleic acids and the liposome-nucleic acid complex is used to facilitate the introduction of the nucleic acids into cells. Lipofectin™ Reagent and LipofectAMINE™ Reagent are commercially available from Life Technologies, Inc., Gaithersburg, MD. Lipofection is carried using either of these reagents according to the manufacturer's protocols.

The term "calcium phosphate co-precipitation" refers to a technique for the introduction of nucleic acids into a cell. The uptake of nucleic acids by cells is enhanced when the nucleic acid is presented as a calcium phosphate-nucleic acid coprecipitate. The original technique of Graham and van der Eb (1973) Virol. 52:456 has been modified by several groups to optimize conditions for particular types of cells. The art is well aware of these numerous modifications. The experimental section herein describes a modification of this technique in Example 1 which is suitable for the introduction of DNA into adherent human cell lines.

The term "Northern Blot" as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp 7.39-7.52)

The term "dot blot" as used herein refers to spotting a sample of containing protein or nucleic acid onto a solid support. The solid support is then probed with a labeled nucleic acid or antibody probe to detect the protein or nucleic acid species of interest (For example, see Sambrook, *supra* at 7.53 for RNA dot blots). Alternatively the reaction products of an assay containing a radioactive substrate can be spotted onto a solid support and the unincorporated substrate washed prior to exposure of the support to X-ray film.

DESCRIPTION OF THE INVENTION

The invention provides novel retroviral vectors having improved promoter function, increased expression from inserted genes and increased packaging efficiency. These novel vectors are suitable for the introduction and expression of genes at high levels in human cells as well as for anti-HIV therapy and general gene therapy applications.

Improved Retroviral Vectors

Most of the currently approved gene therapy protocols utilize amphotropic M-MuLV-based vectors, such as pLNL6 [SEQ ID NO:1; Bender, M.A., *et al.* (1987) J. Virol. 61:1639]. Accumulated experience with this vector has led to the realization that the activity of the M-MuLV LTR is not very strong. In addition, the activity of this LTR in different cell types is unpredictable.

To create an improved retroviral vector suitable for a wide variety of gene expression studies and gene therapy applications, the clinically approved gene therapy vector pLNL6 was modified. The improved vector contains a recombinant LTR which comprises M-MuLV and CMV enhancer elements, two TATA promoters (from HIV and M-MuLV) and the HIV-1 TAR element. The upstream TATA box derived from HIV-1, directs the synthesis of TAR-containing mRNAs. TAR-containing mRNA is responsive to the HIV-1 Tat protein. The downstream TATA box is derived from the M-MuLV, and functions to direct the synthesis of mRNAs lacking the TAR element. This dual-promoter design allows high basal levels of mRNA to be synthesized from the vector sequences in the absence of the Tat protein (*i.e.*, for general gene therapy applications). In the presence of the Tat protein (*i.e.*, in HIV infected cells), this dual promoter is induced and directs the production of high levels of mRNA. This improved vector, termed pLCTSN, directs higher levels of expression from inserted genes than does the parental pLNL6 vector. Therefore, the pLCTSN vector is useful for the delivery of a wide variety of genes, including anti-HIV genes.

To improve the packaging efficacy of these vectors, extended packaging signals and a 3' splice acceptor sequence from the M-MuLV genome were added creating the pLGCTSN vector. These modifications increase the efficiency of packaging the vector RNA into viral particles allowing the production of high-titer recombinant virus stocks. High-titer stocks are needed when the target cells are present in low frequency in the tissue being infected (*i.e.*, bone marrow stem cells). Addition of the extended packaging signal provides for competition between the anti-HIV genomes (provided by the vector) and the HIV genome (present in the infected cell).

These newly engineered retroviral vectors are useful as vectors to allow the introduction and study of genes in human cells. These vectors find utility in the area of the study of mammalian gene expression as well as in general gene therapy applications. For example, the pLCTSN and the pLGCTSN vectors exhibited higher promoter activities in hepatoma cell lines [HepG2 (ATCC HB 8065) and HuH-7 (Nakabayashi, H. *et al.* (1982) Cancer Res.

42:3858)] than the conventional pLNL6 vector. Most importantly, the vector pLCTSN has demonstrated prolonged stability in the hepatoma cells and epithelioid cells [HeLa cells (ATCC CCL 2)]. Some of the obstacles faced by the current gene therapy vectors lie in the poor expression level and the lack of long-term performance. It is contemplated that the vectors of the present invention will be used in therapeutic gene therapy.

Improved Vectors For The Study Of Gene Expression In HIV-Infected Cells

In addition to their use as delivery vehicles of genes for general gene expression studies, the improved vectors, pLCTSN and pLGCTSN are useful for the delivery of genes, including anti-HIV agents, to HIV-infected cells. As discussed above, the novel LTR contained on these vectors contains the HIV-1 TATA box which directs the synthesis of TAR-containing mRNAs. TAR-containing mRNAs are responsive to the HIV-1 Tat protein (present in HIV-infected cells). The novel LTRs direct high levels of mRNAs in the presence of Tat in infected cells. Therefore, the pLCTSN vector is useful for the delivery of genes, including anti-HIV genes, to HIV-infected cells.

Examples of anti-HIV genes include anti-HIV ribozymes [Chang, L.-J. and Stoltzfus, C.M. (1985) Mol. Cell. Biol. 5:2341; Chang, L.-J. and Stoltzfus, C.M. (1987) J. Virol. 61:921; and Sarver, N.E.M. *et al.* (1990) Science 247:1222] and intracellular single chain antibody genes [Marasco, W.A. *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:7889]. Gene sequences encoding anti-HIV genes are inserted into the improved retroviral vectors to yield anti-HIV vectors.

To further improve the therapeutic efficacy of these vectors, packaging sequences from the HIV-1 genome were added. The addition of the HIV-1 packaging signal provides for competition between the anti-HIV genomes (provided by the vector) and the HIV genome (present in the HIV infected cell). This *in vivo* competition results in decreased HIV production by infected cells, as many of the virus particles will contain the anti-HIV genome (*e.g.*, pseudotype particles) rather than the HIV-1 genome. In addition to reducing HIV production, vectors containing HIV-1 packaging signals allow for the access of the therapeutic anti-HIV vector to the target HIV genome.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides novel long terminal repeat (LTR) constructs and improved retroviral vectors. The improved vectors contain novel LTRs which results in vectors having improved promoter function, increased expression of inserted genes and increased packaging efficiency. It is contemplated that these novel vectors will be useful for anti-HIV therapy, as well as other gene therapy applications. Here, the detailed description involves construction of novel vectors through reconstruction of LTRs from approved vectors and various assays utilized to assess their functions.

Reconstruction Of The M-MuLV LTR Using A Clinically Approved Gene Therapy Vector

To improve the expression of genes from existing M-MuLV-based gene therapy vectors, the M-MuLV LTR was modified to include a truncated CMV-IE enhancer element and the HIV-1 TAR element. This modification produces a hybrid promoter which exhibits high basal activity and which is inducible by Tat to higher levels.

The clinically approved retroviral vector pLNL6 was used as a starting point. The retroviral vector pLNL6 is 6145 bp in length, and contains a M-MuLV promoter in the 3' LTR and a murine sarcoma virus (MSV) promoter in the 5' LTR [Bender, M.A., *et al.* (1987), *supra*]. For ease in subsequent cloning steps, the few cloning sites and the internal SV-*neo* gene in pLNL6 were replaced with the synthetic polylinker shown below to generate pLLL. pLNL6 was digested with *Cla*I and *Bcl*I. The digested vector was purified and a double stranded insert containing the polylinker site was inserted. This double stranded insert was made by annealing the following two oligonucleotides together. 5'-GATCTAAGCTTGC GGCCGCAGATCTCGAGCCATGGATCCTAGGCCTGATCACGCGTCGACTCGCGAT-3' (SEQ ID NO:2) and 5' CGATCGCGAGTCGACGCGTGATCAGGCCTAGGATCCATGGCT CGAGATCTGCGGCCGCAAGCTTA-3' (SEQ ID NO:3).

After annealing the above oligonucleotides, the resulting DNA fragment contains restriction sites for *Hind*III, *Not*I, *Bgl*II, *Xho*I, *Nco*I, *Bam*HI, *Avr*II, *Stu*I, *Bcl*I, *Mlu*I, *Sal*I, *Nru*I, and *Cla*I. The digestion of pLNL6 followed by the insertion of the annealed oligonucleotides resulted in the deletion of the sequences present from *Bcl*I (nt 1625) to *Cla*I (nt 3049) in pLNL6 (SEQ ID NO:1).

To obtain a vector carrying a selective marker for cell selection, pLSN was constructed by inserting a fragment from *Bam*HI to *Stu*I of pLNSX containing the SV40 promoter into

the *Bam*HI and *Stu*I sites of pLLL to obtain pLLLSV40. The amplified *neo* gene was isolated by amplifying the *neo* gene using the polymerase chain reaction (PCR). pLNSX was used as the template and the following oligonucleotides were used to amplify the *neo* gene: 5'-AAGCTTGATCACCACCATGATTGAACAAGATGG-3' (SEQ ID NO:4) and 5'-CCGGA TCCGTCGACCCCAGAGTCCCGCTCAGAAG-3' (SEQ ID NO:5). The amplification products were then digested with *Bcl*I and *Bam*HI and cloned into *Bcl*I-digested pLLLSV40 to generate pLSN. The primers used to amplify the *neo* gene contained the modified translation initiation control sequence -CCACCATG [Kozak. M. (1986) Cell 44:283]. The use of this modified translation initiation control sequence greatly increased the strength of the neomycin resistance gene in tissue culture cells (*e.g.*, HeLa, 3T3, and HepG2, data not shown).

To create a vector containing a modified M-MuLV LTR only the 3' LTR was reconstructed, since it would replace the 5' LTR after one round of reverse transcription. Sequences upstream of the M-MuLV TATA box in the U3 of the 3' LTR were modified to include a genetic cassette containing both the CMV-IE enhancer/promoter and the TAR sequence, or the TAR sequence alone.

To achieve this, the 3' LTR from pLNS was isolated by *Cla*I and *Nde*I digestion (corresponds to nucleotides 3049-4082 of pLNL6) and cloned into the pSP72 vector (Promega) between the *Cla*I and *Nde*I sites thus generating a subclone containing only one M-MuLV LTR (pSP72-3'LTR). To pSP72-3'LTR, fragments containing the HIV-1 TAR and a CMV-TAR DNA fragment were inserted by the following series of steps. First, the *Sac*I site near the M-MuLV LTR TATA box (nucleotide 3604 of the pLNL6 numbering system) was changed to an *Eco*RI site by annealing an *Eco*RI adapter (5'-GAATTCAGCT-3'). The HIV-1 TAR fragment (~200bp) was made by PCR using pU3-R-CAT [Chang, *et al.* J. Virol. 67: 743 (1993)] and the following primer pair: 5'-GCATCTAGAGTACTTCAAGAACTGC-3' (SEQ ID NO:6) (this primer corresponds to sequences near the HIV-1 TATA box and provides an *Xba*I site) and 5'-GGGAATTCGAGGCTTAAGCAGTGGGTTCC-3' (SEQ ID NO:7) (corresponds to sequences 3' to the HIV TAR and provides an *Eco*RI site).

The CMV-TAR fragment (~343bp) was made by PCR using dl.kB/Sp1 CMV-IEa U3-R-CAT as the template and a primer pair consisting of: 5'-CCGGAGTAGCTAGCTGGAGTTC CGC-3' (SEQ ID NO:8) (corresponds to sequences located 5' to the CMV-IEa element and provides an *Nhe*I site) and SEQ ID NO:6 (listed above: *i.e.*, the same 3' primer used to generate the TAR fragment). The two amplified fragments were digested with *Xba*I (for the

TAR construct) or *Nhe*I (for CMV-TAR) and *Eco*RI, and cloned into the *Xba*I-*Eco*RI digested modified pSP72-3'LTR (contains an *Eco*RI site in place of the *Sac*I site).

The identities of the two final products pMT and pMCT, were confirmed by restriction enzyme digestion and sequencing. To make the CAT reporter constructs, the cat-SV40 polyA fragment (~1631 bp) was obtained by digesting 5 µg of the pU3-R-CAT with *Hin*dIII and *Bam*HI. The cat-SV40 polyA fragment was gel-purified and the ends were made blunt using T4 polymerase. An *Asp*718 linker [5'-GCTAGCGGTACC-3' (SEQ ID NO:9)] was ligated to the blunt ends and the fragment was cloned into the *Asp*718-digested pMT or pMCT to generate pMT-cat and pMCT-cat.

Generation Of Retroviral Vectors Comprising Recombinant 3'LTRs

Both pMT and pMCT are single LTR plasmids. A four-fragment ligation procedure was used to construct the two-LTR packaging vectors pLCTSN and pLTSN. The vector was pLSN digested with *Sac*II and *Kpn*I. The three fragments inserted sequentially were *Sac*II to *Xho*I of pLSN, *Xho*I to *Nhe*I of pLSN, and *Nhe*I to *Kpn*I of pMCT or pMT.

Further modification of the gene therapy vector included generation of packaging and splicing-signal modified retroviral vectors by insertion of a fragment containing an extended packaging signal (from the *gag* gene) and a 3' splice site (from the *env* gene) from the M-MuLV genome. To accomplish this, a *Spe*I-*Bam*HI fragment of pDGLtax/rex [Akagi, T. *et al*. Gene 106: 255 (1991)] was cloned into pLCTSN digested with the same set of enzymes as used above to generate pLGCTSN.

Improved Efficiency of Promoter Activity in Human Cells

Promoter activities of the modified LTR in the presence or absence of the HIV-1 Tat were tested by transfecting the CAT reporter constructs described above into a panel of human cell lines. The level of CAT enzyme activity produced in the transfected cell lines is used to compare the relative strength of different promoter constructs. These cell lines included hepatoma cell lines, T and B lymphoid cell lines and an epithelioid cell line. The pMCT construct exhibited high promoter activity in the absence of Tat. In the presence of Tat, the pMCT LTR was trans-activated significantly (2 to 16-fold increase in activity seen in the presence of Tat).

On the other hand, the M-MuLV-TAR (pMT) construct was not found to be ideal because it demonstrated low basal activity (*i.e.*, activity in the absence of Tat) and was not

responsive to Tat. As shown in Example 4 (Table 1), in comparison with an already strong CMV-IE promoter, the MuLV-CMV-TAR (pMCT) promoter exhibited a 2-fold higher basal activity and was further activated 4-5 times when Tat was present as judged by the amount of CAT activity present in the transfected cell lines.

These results indicate that the M-MuLV LTR and HIV TAR combination (pMT) is not sufficient to make the M-MuLV LTR Tat-responsive, but Tat can trans-activate the pMCT promoter. Significantly, the CMV-TAR modification renders the M-MuLV promoter responsive to the HIV-1 Tat.

This conclusion was yet further supported by assays using a T-lymphoid cell line CEM-TART [Chen, H. *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:7678] in which the Tat protein is expressed constitutively. CEM-TART cells were electroporated with a series of plasmid DNAs and the CAT reporter gene expression was determined. The result of this study indicated that the pMCT promoter exhibited the highest activity among the M-MuLV LTR, CMV-IE and HIV-1 LTR promoters in the human cell lines tested. Therefore, for the purpose of targeting HIV-infected cells, the pMCT construct appears to be ideal, since it exhibits high activity in the absence of Tat, and is strongly activated when Tat is present (*i.e.*, during HIV infection).

In summary, pMCT was found to exhibit promoter activity at levels higher than the pLLL (M-MuLV promoter itself) in most of the human cell lines tested and much better than the pLLL in some hepatoma (HepG2, HuH-7) and lymphoid cell lines (H9, CEM, AA2, CEM-TART). The pMCT promoter was trans-activated by HIV-1 Tat in all the human cell types tested. Thus, the newly designed vector appears to be very useful for anti-HIV and general gene therapy applications as well as for the expression of genes in human cell lines.

Determination Of The Transcription Initiation Sites In The Modified LTR

Preliminary studies indicated that the modified CMV/TAR M-MuLV LTR, which worked well in human cells, is less active than the native M-MuLV LTR in mouse cells (*e.g.*, 3T3). It is possible that the double TATA-box feature does not work well in mouse cells. In general, for the selection of packaging producer cell clones, ecotropic virus infection is preferred to DNA transfection. The poor expression level of the recombinant CMV/TAR M-MuLV LTR in mouse cells limits the use of the ecotropic infection method because the modified 3' LTR, which is to be reverse transcribed and relocated to the 5' end, would not be active in mouse cells. Nonetheless, long-term producer cells (PA317) have been generated by

DNA transfection and have not had problems with stability (producing virus having average titers of 10^4 to 10^5 per ml, see Example 7, Table 3).

Because the CMV/TAR M-MuLV LTR construct contains both the MuLV TATA box and the HIV TATA box, it is possible that both direct transcription initiation. To determine the transcription initiation sites of the new vector, the initiation sites of mRNA from HepG2 cells transfected with pMCT-cat plasmid were analyzed by RNase mapping. Cytoplasmic polyA$^+$ RNA from transfected HepG2 cells was harvested and analyzed by Northern blotting and RNA protection. As discussed in Example 5, the results indicated that both HIV and M-MuLV transcription initiation sites were used, but the upstream HIV transcription initiation site was preferred to the downstream M-MuLV initiation site. In addition, quantitation of the relative amounts of RNA produced from the pMCT promoter in the presence or absence of Tat confirmed that the pMCT transcription was trans-activated by Tat (See Example 5, Figures 14 and 15).

Improved Packaging Efficiency

In addition to the generation of improved promoters, the ability to increase the vector titer of the packaging cells is yet another key issue in retroviral gene therapy. Most clinical gene transfer studies involve infection of hematopoietic stem cells and therefore require high titers of recombinant virus. Efficient stem cell transduction requires the use of viral stocks having a titer of at least 10^6 viral particles/ml.

To improve the packaging efficiency of the traditional vector, an additional M-MuLV packaging signal was cloned into the vector. Along with the extra packaging signal, a 3' splice site was also included. This modification stabilized transcripts expressed by the vector and enhance its packaging efficiency. The packaging efficiencies of these new vectors, including pLSN, pLCTSN and pLGCTSN, were studied using the PA317 packaging cells (Example 7). Vector DNA was introduced into PA317 cells using lipofectamine (BRL). Culture supernatant containing the packaged vector RNA from the transfected cells was removed and used to infect HeLa or Huh7 cells. The infected cells were cultured in medium containing the antibiotic G418 (all three vectors contain the *neo* gene and therefore confer resistance to G418 upon the infected cell). The number of G418-resistant colonies produced per ml of culture supernatant used gives the titer or packaging efficiency of the vector in PA317 cells. The results showed that the packaging efficiency of pLGCTSN, which contained an additional packaging signal, increased about 3-5 fold over the others (pLNL6,

pLSN. or pLCTSN). In addition. expression of the new vector (pLGCTSN) was consistently 3-10 times better than the pLCTSN or the pLSN constructs as judged by CAT assays [All three vectors contain the CAT gene: therefore. the level of CAT activity produced from a vector can be used to determine the activity of the LTR on a given vector relative to another vector(s) in CAT assays].

Long-Term Stability Of Gene Expression From The Novel Vectors

The long term stability of the modified vector was studied in HeLa and HepG2 cells. Cells infected with pLSN. pLCTSN. or pLGCTSN were selected by growth in the presence of G418 (resistant colonies took about two weeks to establish). Selected [*i.e.*. G418-resistant (G418ʳ)] cells were then assayed for CAT activity. Results of this study showed that CAT expression from pLCTSN was more stable than CAT expression from pLGCTSN in HeLa and HepG2 cells. In addition. the level of CAT expression of pLCTSN was also 2-3 fold higher than the parental construct pLSN as judged by CAT assays on HeLa cells 1 to 2 months after transduction with these vectors. Stable expression of CAT from the pLGCTSN vector was achieved in stably transduced HeLa cells two months after transduction. The pLGCTSN vector also remained responsive to Tat trans-activation two months after introduction of the vector into HeLa cells. Thus. both the pLCTSN and pLGCTSN vectors permit the long term expression of inserted genes in human cells: furthermore this expression remains responsive to induction by the Tat protein.

Addition of HIV-1 Packaging Sequences to the Improved Vectors

Traditional gene therapy vectors can only infect the target cell once because of the lack of M-MuLV structural proteins in the target cells. To further modify the gene therapy vector for anti-HIV purposes. sequences in HIV that are essential to genome packaging were cloned into pLLLgpt (described in Example 2. *infra*). Two synthetic packaging sequences were designed based upon the consensus sequence derived from several HIV-1 isolates [sequences of the isolates and the consensus sequence were from *Human Retroviruses and AIDS 1993*. I-II. Myers. G.. *et al*. eds. (1993) Theoretical Biology and Biophysics. Los Alamos. NM]. PAK 100 (SEQ ID NO: 10) contains approximately 100 nucleotides derived from HIV-1 and a restriction recognition site for *Bam*HI at the 3' end and a cohesive overhang at the 5' end for *Sal*I. PAK 140 (SEQ ID NO:11) contains approximately 140 nucleotides derived from HIV-1 and overhanging ends compatible with *Bam*HI and *Sal*I.

The presence of PAK140 sequences on vector RNA reduces the production of infectious HIV particles in a cell expressing both the HIV genome and the PAK-containing vector genome.

The sequences contained in either PAK100 or PAK140 do not appear in any naturally occurring isolate of HIV-1; they contain mutated splice donor sequences and PAK140 replaces sequences near the ATG of the *gag* gene with sequences not found in known HIV isolates. These sequences are derived from but are not identical to the consensus sequence derived from a comparison of several HIV-1 isolates.

Upon transduction, expression of HIV packaging proteins in the target cells (HIV-infected) allows packaging of the M-MuLV vector containing the HIV-1 packaging sequences (PAK100 or PAK140) into HIV particles. This design can potentially expand the therapeutic efficacy of the anti-HIV antisense and ribozyme vectors by both reducing the amount of viral particles containing the HIV genome produced by the infected cell and by allowing access of the therapeutic anti-HIV vector to the target HIV genome. These packaging sequences are cloned into the vector pLCTSN, which is then transfected into HIV-infected cells to determine if the vector sequences are packaged into HIV particles.

The HIV-1 packaging sequences (*e.g.*, PAK140) were inserted into several retroviral vectors including pLGCTSN (the resulting vector was termed pLGCTSN-PAK140). pLGCTSN-PAK140 or other vectors were then transduced into either H9 or AA2 cells and the transduced cells where then infected with HIV-1 or HIV-2 to examine the ability of this vectot to protect cells from HIV infection.

The presence of the pLGCTSN-PAK140 vector was found to protect AA2 or H9 cells from infection by HIV. These studies demonstrate that the novel anti-HIV vector pLGCTSN-PAK140 can block infection by HIV-1 and HIV-2 up to 100% in different human CD4 lymphoid cell lines for longer than 40 days. The ability of cells containing the pLGCTSN-PAK140 vector to block invention by HIV was compared with cells containing a conventional MLV vector, pLSN, which either lacked (*i.e.*, pLSNnef) or contained (*i.e.*, pLSN-PAK140) the PAK140 sequences on the vector. The results obtained clearly indicated that the further modifications of the vector in the LTR and the packaging (psi) regions contained within pLGCTSN-PAK140 are critical for the anti-HIV effect displayed by pLGCTSN-PAK140 as the presence of pLSN-PAK140 in H9 or AA2 cells had no effect upon the ability of HIV-1_{NL4-3}, HIV-1_{ELI} or HIV-2 ROD to infect these cells (*See* Figures 26 and 27). Thus, the novel Tat-inducible LTR and the extended packaging sequences (*i.e.*, extended packaging signal)

and the intron contained on the pLGCTSN vector provide features necessary for the generation of a successful anti-HIV gene therapy vector.

While not limiting the invention to any particular theory, the inhibitory effects of pLGCTSN-PAK140 may occur at the transcriptional level where competition for Tat may reduce HIV expression; alternatively the inhibitory effects may occur at the level of virion assembly as the pLGCTSN-PAK140 transcript may compete for packaging into the HIV particle. In addition, the pLGCTSN vector was found to express vector-encoded genes at higher levels in comparison to the use of conventional MLV vectors (*e.g.*, pLSN). In addition, expression of genes from the pLGCTSN vector is more stable in comparison to expression of genes contained within conventional MLV vectors (*e.g.*, pLSN).

The pLGCTSN-PAK140 RNA can be packaged into HIV particles. The resulting chimeric particles (*i.e.*, pLGCTSN-PAK140 RNA inside the HIV viral coat) released from the HIV-infected cell are non-infectious; these chimeric particles may be immunogenic (*i.e.*, capable of eliciting an immune response directed against HIV proteins). Therefore the use of the pLGCTSN-PAK140 vector would be advantageous in HIV gene therapy protocols. Peripheral blood lymphocytes or $CD34^+$ enriched lymphocytes from HIV-infected patients are isolated and transduced with the pLGCTSN-PAK140 virus; the transduced lymphocytes are then infused into the patient. The transduced cells may show resistance to infection by HIV and if already infected, the transduced cells may suppress replication of HIV. The small population of lymphocytes transduced *in vivo* (due to transduction of lymphocytes by the chimeric viral particles) may expand as a percentage of the total lymphocytes in the patient due to the rapid killing of HIV-infected cells due to the cytopathetic effects of HIV. Therefore, even if transduction of human lymphocytes by the novel anti-HIV gene therapy viruses of the present invention occurs with low efficiency, this may not present problems for HIV gene therapy. In addition, minor effects on HIV replication *in vivo* may have a great impact upon development of disease in the patient long term.

The novel anti-HIV gene therapy vectors of the present invention (*e.g.*, pLGCTSN, pLGCTSN-PAK140) may be modified to permit the insertion of other anti-HIV genes into these vectors to permit even greater effects upon the ability to block HIV replication in cells containing these vectors.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); µM (micromolar); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); *g* (gravity); gm (grams); mg (milligrams); µg (micrograms); pg (picograms); L (liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); hr (hour); min (minute); msec (millisecond); °C (degrees Centigrade); AMP (adenosine 5'-monophosphate); cDNA (copy or complimentary DNA); DTT (dithiotheritol); ddH_2O (double distilled water); dNTP (deoxyribonucleotide triphosphate); rNTP (ribonucleotide triphosphate); ddNTP (dideoxyribonucleotide triphosphate); bp (base pair); kb (kilo base pair); TLC (thin layer chromatography); tRNA (transfer RNA); nt (nucleotide); VRC (vanadyl ribonucleoside complex); RNase (ribonuclease); DNase (deoxyribonuclease); poly A (polyriboadenylic acid); PBS (phosphate buffered saline); OD (optical density); HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]); HBS (HEPES buffered saline); SDS (sodium dodecyl sulfate); Tris-HCl (tris[Hydroxymethyl]aminomethane-hydrochloride); rpm (revolutions per minute); ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 25 µg/ml bovine serum albumin, and 26 µM NAD+, and pH 7.8); EGTA (ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid); EDTA (ethylenediaminetetracetic acid); ELISA (enzyme linked immunosorbant assay); LB (Luria-Bertani broth: 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter, pH adjusted to 7.5 with 1N NaOH); superbroth (12 g tryptone, 24 g yeast extract, 5 g glycerol, 3.8 g KH_2PO_4 and 12.5 g, K_2HPO_4 per liter); DMEM (Dulbecco's modified Eagle's medium); ABI (Applied Biosystems Inc., Foster City, CA); Amersham (Amersham Corporation, Arlington Heights, IL); ATCC (American Type Culture Collection, Rockville, MY); Beckman (Beckman Instruments Inc., Fullerton CA); BM (Boehringer Mannheim Biochemicals, Indianapolis, IN); Bio-101 (Bio-101, Vista, CA); BioRad (BioRad, Richmond, CA); Brinkmann (Brinkmann Instruments Inc. Wesbury, NY); BRL, Gibco BRL and Life Technologies (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD); CRI (Collaborative Research Inc. Bedford, MA); Eastman Kodak (Eastman Kodak Co., Rochester, NY); Eppendorf (Eppendorf, Eppendorf North America, Inc., Madison, WI); Falcon (Becton Dickenson Labware, Lincoln Park, NJ); IBI (International Biotechnologies, Inc., New Haven, CT); ICN (ICN Biomedicals,

Inc., Costa Mesa, CA); Invitrogen (Invitrogen, San Diego, CA); New Brunswick (New Brunswick Scientific Co. Inc., Edison, NJ); NEB (New England BioLabs Inc., Beverly, MA); NEN (Du Pont NEN Products, Boston, MA); Nichols Institute Diagnostics (Nichols Institute Diagnostics, San Juan Capistrano, CA); Pharmacia (Pharmacia LKB Gaithersburg, MD); Promega (Promega Corporation, Madison, WI); Stratagene (Stratagene Cloning Systems, La Jolla, CA); UVP (UVP, Inc., San Gabreil, CA); USB (United States Biochemical Corp., Cleveland, OH); and Whatman (Whatman Lab. Products Inc, Clifton, NJ).

Unless otherwise indicated, all restriction enzymes were obtained from New England Biolabs and used according to the manufacturers directions. Unless otherwise indicated, synthetic oligonucleotides were synthesized using an ABI DNA synthesizer, Model No. 391.

EXAMPLE 1

The Wild Type MuMLV LTR Promoter Is A Relatively Weak Promoter In Human Cells

Most of the currently approved gene therapy protocols utilize the amphotropic M-MuLV vector, pLNL6. Accumulated experience with this vector has led to the realization that the activity of the M-MuLV LTR is weak compared to other enhancer/promoters or LTRs. In addition, the activity of this LTR in different cell types was found to be unpredictable. In order to design an improved retroviral vector, the relative strengths of several promoters were examined.

In this example, the M-MuLV LTR, CMV-IE and the activated HIV-1 LTR promoters were tested for their activities using a CAT reporter gene assay. Plasmids containing each of these three promoters directing the CAT gene were transfected into the human HeLa cell line (ATCC CCL 2). CAT activity was measured in order to determine the relative strength of these three promoters. CAT activity is expressed as the percentage of chloramphenicol converted to acetylated forms of chloramphenicol.

Plasmid DNA containing M-MuLV LTR directing the expression of the CAT gene (M-MuLV LTR-cat) was constructed by ligating the vector, pSP72-3'LTR digested with *Asp*718 (BM), to a DNA fragment containing the cat-SV40 polyadenylation site. To achieve this, the 3' LTR from pLNSX was isolated by *Cla*I and *Nde*I digestion (corresponds to nucleotides 3049 to 4082 in pLNL6). This fragment was inserted into pSP72 (Promega) digested with *Cla*I and *Nde*I thereby generating a subclone containing only one M-MuLV LTR

(pSP72-3'LTR). The cat-SV40 DNA fragment was prepared by digesting pU3-R-CAT [Chang, L.-J. *et al.*, (1993) J. Virol. 76:743] with *Hin*dIII and *Bam*HI. The ends of the molecule were then filled in using T4 polymerase (NEB). An Asp718 linker [5'-GCTAGCGGTACC-3' (SEQ ID NO:9)] was ligated to the blunt ends using T4 DNA ligase (IBI) according to the manufacture's protocols.

The plasmid pCMV-cat contains the CMV-IE promoter directing the expression of the CAT gene [Hunninghake, G.W. *et al.* (1989) J. Virol. 63:3026]. The plasmid pU3-R-CAT [Chang, L.-J. *et al.*, J. Virol. 76:743 (1993)] contains the HIV-1 LTR directing the expression of the CAT gene.

Plasmid DNA was purified using an alkaline-SDS, cesium chloride gradient protocol [Sambrook, J. *et al.* (1989) Molecular Cloning: A Laboratory Manual, Vol. 2, pp. 1.21-1.52]. Briefly, bacteria [typically DH5α cells (BRL)] containing the desired plasmid were grown in 150 ml of superbroth in 250 ml flasks overnight in a 37°C environment shaker (New Brunswick). The bacteria were pelleted by spinning at 5.000 rpm for 10 min using a JA10 or JA14 rotor (Beckman). The pellet was resuspend in 5 ml of lysis buffer [50 mM glucose or 15% sucrose W/V, 25 mM Tris (pH 8.0), 10 mM EDTA with 5 mg/ml lysozyme at 4°C] and then incubated on ice for 10 to 20 min. To this mixture, 10 ml of freshly made 0.2 N NaOH, 1% SDS (in ddH_2O) was added and mixed immediately by swirling and rotating the bottle. The bottle was then incubated at room temperature for 5 min. Then, 7.5 ml of cold 7.5 M NH_4OAc (pH 7.5) was added and mixed by swirling the bottle. The mixture was incubated on ice for 5 min. The lysate was then centrifuged in a Beckman JA10 rotor at 8000 rpm for 10 min. The supernatant was transferred into a 50 ml polypropylene tube containing 25 µl of RNase A (10 mg/ml), mixed and incubated at 37°C water bath for 1 hr. The mixture was extracted with a half volume of ddH_2O-saturated phenol (approximately 10 ml) and a half volume of chloroform by shaking vigorously for 1-2 min. The aqueous and organic phases were then separated by centrifugation at 800 x g for 5 min in a table top centrifuge (Beckman). The top layer was mixed with 0.6-1 volume of cold 2-propanol and incubated at -20°C for at least 30 min.

The plasmid DNA was pelleted by centrifugation at 10.000 rpm in a JA20 rotor (Beckman) for 20 min. The pellet was then dried in a vacuum. The pellet was resuspended in 1.5 ml of TE (10 mM Tris, pH 8.0, 1 mM EDTA), then mixed with 3 ml of CsCl solution (1.2 g/ml, ref. index 1.4155, prepared with autoclaved ddH_2O and filtered through a 0.45µ filter) and transferred into a Beckman VTi 65 tube. One hundred microliters of EtBr stock (5

mg/ml) was then added. The tube was filled to the sub-neck level with CsCl:ddH$_2$O (2:1). The weight of the tube was approximately 9.5 grams. The tube was sealed and centrifuged in a Beckman VTi 80 rotor at 60.000 rpm for at least 4 hr at 19°C.

The plasmid DNA (the lower band in the gradient) was drawn off using a 21 gauge needle attached to a 1 ml syringe. The plasmid band was extracted three times with 1 ml of 5 M NaCl-saturated 2-propanol. and 4 ml of ddH$_2$O and 5 ml of cold iso-propanol were added. The plasmid DNA was precipitated at -20°C overnight. The DNA was pelleted by centrifugation at 10.000 rpm using a Beckman JS13 rotor for 30 min. The pellet was rinsed with 70% ethanol carefully and dried under vacuum. The DNA was resuspended in 400 µl of ddH$_2$O. The DNA concentration was determined by measuring the absorption at 260 nm in a spectrophotometer. The concentration of the plasmid DNA was also confirmed by running an aliquot on a 1% agarose gel followed by staining with 0.05 mg/ml of EtBr.

Plasmids containing the M-MuLV LTR. the HIV-1 LTR. and the CMV-IE enhancer/promoter were used to transfect HeLa cells in 6-well plates (Falcon) using a modification of the original $Ca_3(PO_4)_2$-DNA coprecipitation procedure (Graham. F.L. and van der Eb. A.J., Virol., 52:456 [1973]). Briefly, approximately 4 x 10^5 HeLa cells were plated onto 6 well plates 20 hours prior to the addition of the DNA precipitate. The HeLa cells were at approximately 80-90% confluency when the DNA was added. HeLa cells were grown in DMEM (BRL) containing 10% FBS (BRL) and penicillin and streptomycin (BRL) and were fed with 2 ml of fresh DMEM containing 10% FBS and antibiotics 1 hour before addition of the DNA precipitate.

The DNA precipitates were made by mixing 90 µl of ddH$_2$O containing 2 µg of the desired CAT reporter plasmid [0.2 µg of pCEPtat (described in Example 2) was also added to the tube containing pU3-R-CAT in order to activate the HIV-1 promoter]. 10 µl of 2.5 M $CaCl_2$, and 100 µl of 2x BES buffer (50 mM N. N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid. 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95). Each well contained 2 ml of media and 200 µl of the DNA precipitates and was incubated in an atmosphere containing 5% CO_2 overnight at 37°C. The next day. the cells were washed once with growth medium (DMEM with 10% FBS) and fed with 2 ml of fresh media. Cell lysates were prepared 48 hr after changing the medium and CAT enzyme assays were performed.

Each transfection included 0.1µg of the pXGH5 plasmid (Nichols Institute Diagnostics) which allows the transfected cells to express human growth hormone into the culture supernatant. Quantitation of the human growth hormone was performed using the

commercially available ELISA kit provided by Nichols Institute Diagnostics. This provided an internal control for transfection experiments.

CAT assays were performed as described [Chang, L.-J. *et al.*, (1993) J. Virol. 76:743]. Briefly, the HeLa cells were harvested 60 hr after the addition of the DNA, washed three times in PBS and subjected to three cycles of freeze-thawing in a 37°C water bath and a dry-ice ethanol bath. The protein concentration in the cell lysates was determined by using a DC protein assay kit (BioRad). To obtain results within the linear kinetic range of CAT activity, the amount of cell lysate used in each reaction was adjusted to give a detectable signal within 1 hr and less than 60% consumption of the input substrate [^{14}C]chloramphenicol (0.5 μCi: 55 mCi/mmol: ICN). The enzyme concentration was determined by a serial dilution for lysates with high levels of CAT activity.

Following the incubation of the cell lysate and the substrate, the reaction products were spotted onto a TLC plate and chromatographed in a solution containing 95% chloroform and 5% methanol for 45 min. The plates were allowed to dry and then were autoradiographed by exposing the plates to photographic film for 12 hr at room temperature. The amount of chloramphenicol present in acetylated or non-acetylated forms was quantitated by exposing the TLC plates to an imaging plate for 2 hr and scanning with a phosphoimager (Model BAS 1000, Fuji Medical Systems, USA Inc.).

Figure 2 shows the conversion of chloramphenicol to acetylated forms of chloramphenicol by the CAT enzyme produced by plasmids containing the HIV, CME-IE and MLV promoters. The amount of CAT enzyme present in the transiently transfected HeLa cells is a function of the strength of the enhancer/promoter which drives the expression of the CAT gene. Figure 2 shows that the M-MuLV LTR is 9 times less active than the HIV LTR and 11 times less active than the CMV-IE enhancer promoter in HeLa cells. Clearly the M-MuLV LTR is not the optimal control signal when the target cell is of human origin (as will be the case for all gene therapy applications).

EXAMPLE 2

The Use Of Internal Promoters In Retroviral Vectors Is Disadvantageous

The results shown in Figure 2 indicate that the M-MuLV LTR is not a strong promoter in human cells. In an attempt to achieve higher levels of expression of genes carried on a M-MuLV vector internal promoters have been utilized. The internal promoter is placed

downstream of the viral LTR and is used to drive the expression of the inserted gene(s). However, as shown below, the activity of the internal promoter is often significantly reduced due to interference from the upstream M-MuLV promoter.

The strength of an internal promoter was compared with several heterologous promoters using a Tat trans-activation assay. HeLa cells were transfected with a series of plasmids which contains the *tat* gene driven by a given enhancer/promoter as shown schematically at the bottom of Figure 8. In these constructs, the *tat* gene is driven by either no promoter (pSP72tat), the SV40 promoter (pSV-tat), the M-MuLV LTR driving the *gpt* gene followed by an internal SV40 promoter driving the *tat* gene (pLLLgptSVtat), the CMV-IE promoter (pCEP-tat) or the RSV LTR (pREP-tat). All cells are co-transfected with a second plasmid containing the HIV LTR (pU3-R-cat) driving the CAT gene. The HIV LTR is induced or trans-activated by the Tat protein. Thus the strength of the various promoters can be measured by determining the amount of CAT enzyme produce by the activated HIV LTR.

The no promoter control plasmid (pSP72tat) was made by cloning the *tat* gene into pSP72 (Promega). The *tat* gene was isolated using PCR from the plasmid pSV-tat [Peterlin, B. M. *et al.* Proc. Natl. Acad. Sci. USA 83:9734 (1986)]. The primers used to amplify the *tat* gene were 5'-AAGGATCCTCGAGCCACCATGGAGCCAGTAGATCCT-3' (SEQ ID NO:12) and 5'-CAAGATCTGCATGCTAATCGAACGGATCTGTC-3' (SEQ ID NO:13). Reaction conditions were as described [Chang, L.-J. *et al.* (1993) J. Virol. 67:743]. Briefly, *Pfu* polymerase (Stratagene) was used according to the manufacturer's instructions in a 50 µl reaction containing 0.5 µg of each primer, 0.01 µg of pSVtat [Peterlin, B.M. *et al.* (1986) Proc. Natl. Acad. Sci. USA 83:9734] for 30 cycles under the following conditions: step 1: 94°C for 5 min; step 2: 50°C for 1 min; step 3: 72°C for 1 min; step 4: 92°C for 1 min and step 5: repeat steps 2-4 for 30 cycles. The *tat* gene was recovered from the PCR products by digestion with *Bam*HI and *Bgl*II and inserted into pSP72 (Promega) digested with *Bam*HI and *Bgl*II to generate pSP72tat.

pCEP-tat (contains the CMV-IE promoter driving the *tat* gene) and pREP-tat (contains the RSV LTR driving the *tat* gene) were constructed as follows. pSP72tat was digested with *Xho*I and *Bam*HI to isolate the *tat* gene. This *Xho*I/*Bam*HI fragment was then inserted into either the eucaryotic expression vector pCEP4 or pREP4 (Invitrogen) to generate pCEP-tat and pREP-tat, respectively. *Pfu* polymerase (Stratagene) was used in place of *Taq* DNA polymerase in the PCR because of its lower error rate. PCR conditions were as described above.

pLLLgptSVtat (the M-MuLV/SV40 construct) was made as follows. pLLL was constructed by digestion of pLNL6 with *Cla*I and *Bcl*I. The digested vector was purified and a double stranded insert containing a polylinker site was inserted. This double stranded insert was made by annealing the following two oligonucleotides together. 5'GATCTAAGCTTGC GGCCGCAGATCTCGAGCCATGGATCCTAGGCCTGATCACGCGTCGACTCGCGAT-3' (SEQ ID NO:2) and 5'-CGATCGCGAGTCGACGCGTGATCAGGCCTAGGATCCATGGCT CGAGATCTGCGGCCGCAAGCTTA-3' (SEQ ID NO:3). After annealing the above oligonucleotides, the resulting DNA fragment contains restriction sites for *Hind*III, *Not*I, *Bgl*II, *Xho*I, *Nco*I, *Bam*HI, *Avr*II, *Stu*I, *Bcl*I, *Mlu*I, *Sal*I, *Nru*I and *Cla*I. The digestion of pLNL6 followed by the insertion of the annealed oligonucleotides resulted in the deletion of the sequences located between nucleotides 1625 (*Bcl*I site) and 3049 (*Cla*I site) in pLNL6. pLLL is shown schematically in Figure 3.

pLLLSV40 was then constructed by inserting a fragment containing the SV40 promoter (isolated by digesting pLNSX with *Bam*HI and *Stu*I) into pLLL digested with *Bam*HI and *Stu*I. This generated pLLLSV40 (shown schematically in Figure 4).

The *tat* gene was amplified as described above, digested with *Bam*HI and *Bgl*II, and cloned into the *Bcl*I site of pLLLSV40 to generate pLLLSVtat (pLLLSVtat is shown schematically in Figure 5).

The *gpt* gene was amplified by PCR from pMSG (Pharmacia) using primers comprising sequences 5'-ATCTAGAAGCTTAGTGCGCCAGATCTCTATAATC-3' (SEQ ID NO:14) and 5'-ATCTAGACTCGAGTTAGCGACCGGAGATTGGC-3' (SEQ ID NO:15). The PCR products were digested with *Hind*III and *Xho*I and cloned into pSP72 (Promega) digested with *Hind*III and *Xho*I to generate pSP72gpt.

pLLLgpt (shown schematically in Figure 6) was generated by cloning the *gpt* from pSP72gpt (*Hind*III to *Xho*I) into *Hind*III and *Xho*I digested pLLL. Then, the SVtat fragment was isolated from pLLLSVtat by digestion with *Bam*HI and *Sal*I: this fragment was inserted into pLLLgpt digested with *Bam*HI and *Sal*I to generate pLLLgptSVtat (pLLLgptSVtat is shown schematically in Figure 7).

HeLa cells were transfected with 0.1 μg of a heterologous promoter plasmid driving the *tat* gene and 2 μg of the pU3-R-cat plasmid which contains the HIV LTR driving the CAT gene (the reporter gene). Plasmid purification, transfections and CAT assays were performed as described in Example 1. Figure 8 is a simple schematic of the organization of the plasmids used in this Example. The following abbreviations are used in Figure 8: X, pSP72tat (the no

promoter control plasmid): SV40. pSVtat: M-MuLV/SV40. pLLLgptSVtat: CMV. pCEP-tat and RSV. pREP-tat.

Figure 9 depicts the results of CAT assays performed on extracts made from HeLa cells transfected with either pSP72tat. pSVtat. pLLLgptSVtat. pCEP-tat or pREP-tat. As shown by the percentage conversion data in Figure 9. the presence of the M-MuLV LTR upstream of the SV40 enhancer/promoter reduced the activity of the SV40 promoter approximately two-fold (compare lanes 2 and 3: 10.3% conversion of chloramphenicol when only the SV40 promoter is present on the *tat* plasmid compared to 5.4% conversion when the M-MuLV LTR is present upstream of the SV40 promoter). Figure 9 also shows that the SV40 promoter is 6-13 fold less active than the CMV promoter in human cells (compare lanes 2.3 and 4). The RSV LTR (lane 5) was about 50% as strong as the CMV promoter in HeLa cells.

The results shown in Figures 2 and 9 indicate that the promoter activity of the M-MuLV LTR is not comparable in strength to an activated HIV-1 LTR or to the CMV-IE promoter. Furthermore. these results demonstrate that the use of an internal promoter in the M-MuLV vector is disadvantageous. as the activity of the downstream promoter is reduced. Therefore an improved LTR was designed to overcome the inherent limitations of the M-MuLV LTR without the use of an internal promoter.

EXAMPLE 3

Reconstruction Of The M-MuLV LTR To Increase Promoter Activity

The HIV-1 LTR contains a very strong promoter which is active in almost all human cell types when the viral trans-activator Tat is present. The genetic element in HIV which mediates Tat activation is termed TAR (Tat-activation response). The TAR element is located in the U5 region of the HIV LTR. The TAR RNA physically binds to the viral trans-activator Tat to mediate the trans-activation function of Tat [Vaishnav. Y.N. and Wong-Staal. F. (1991) Ann. Rev. Biochem. 60:577].

A series of heterologous enhancer/promoter hybrids in the HIV-1 LTR in connection with TAR were previously constructed [Chang. L.-J. *et al.*. (1993) J. Virol. 67:743]. These studies revealed that the combination of the CMV-IE enhancer/promoter and the HIV-1 TAR element creates a hybrid promoter which exhibits high basal activity (*i.e.*. the activity of the hybrid promoter in the absence of Tat is higher than that of the wild type HIV-1 LTR) and which is inducible by Tat to higher levels (*i.e.*. the hybrid promoter is activated or induced by

Tat). These results showed that the activity of the HIV-1 LTR could be increased by substituting the CMV-IE enhancer/promoter for a portion of the HIV-1 LTR (the portion containing the NF-kB and Sp1 binding sites) while maintaining Tat responsiveness.

In order to create a novel LTR which is much stronger than the endogenous M-MuLV LTR and is responsive to Tat, the M-MuLV LTR was modified to include a truncated CMV-IE enhancer element and the HIV-1 TAR element (This recombinant M-MuLV LTR is present in the pMCT-cat construct described below).

To reconstruct the MuLV LTR, a reporter plasmid, pSP72-3'LTRcat, was generated. pSP72-3'LTRcat contains the 3' LTR from the pLNL6 vector [Miller, A.D. and Buttimore, C. Mol. Cell. Biol. 6:2895 (1986); Bender, M.A. *et al.*, J. Virol. 61:1639 (1987) and Miller, A.D. and Rosman, G.J. BioTechniques 7:980 (1989)], the CAT gene and a SV40 polyadenylation site. pSP72-3'LTRcat was constructed as follows. The 3' LTR from pLLL was isolated by *Cla*I and *Nde*I digestion (corresponds to nucleotides 3049-4086 in pLNL6). The 3' LTR fragment was then cloned into pSP72 (Promega) digested with *Cla*I and *Nde*I to generate pSP72-3'LTR. pSP72-3'LTR contains only one M-MuLV LTR.

pSP72-3'LTRcat was then further modified to create pMT-cat and pMCT-cat. The LTR present in pMT-cat replaces the CAAT box upstream of the M-MuLV TATA box with the HIV-1 TATA/TAR. The LTR present in pMCT-cat replaces the CAAT box upstream of the M-MuLV TATA box with the CMV-IE enhancer plus the HIV-1 TATA/TAR. These constructs are depicted schematically in Figure 10.

To generate pMT-cat and pMCT-cat, fragments containing the HIV-1 TAR and a CMV-TAR DNA fragment were inserted into pSP72-3'LTR as follows. First, the *Sac*I site near the M-MuLV LTR TATA box (corresponds to nucleotide 3604 in the pLNL6 numbering system) was changed to an *Eco*RI site by annealing an *Eco*RI adapter (5'-GAATTCAGCT-3') to the *Sac*I ends. The HIV-1 TAR fragment (approx. 200 bp) was isolated from pU3-R-CAT using the PCR and the following primer pair: 5'-GCATCTAGAGTACTTCAAGAACTGC-3' (SEQ ID NO:6) (this primer corresponds to sequences near the HIV-1 TATA box and provides an *Xba*I site) and 5'-GGGAATTCGAGGCTTAAGCAGTGGGTTCC-3' (SEQ ID NO:7) (corresponds to sequences 3' of the HIV-1 TAR and provides an *Eco*RI site).

The CMV-TAR fragment (approx. 343 bp) was isolated from dl.kB/Sp1 CMV-IEaU3-R-CAT using the PCR and a primer pair consisting of: 5'-CCGGAGTAGCTAGCTGGAGTTC CGC-3' (SEQ ID NO:8) (corresponds to sequences located 5' to the CMV-IEa element and provides a *Nhe*I site) and SEQ ID NO:7 (listed above; *i.e.*, the same primer used to generate

the TAR fragment). The two amplified fragments were digested with *Xba*I (for the TAR construct) or *Nhe*I (for CMV-TAR) and *Eco*RI and cloned into the modified pSP72-3'LTR (contains an *Eco*RI site in place of the *Sac*I site) digested with *Xba*I and *Eco*RI.

The identities of the two final products, pMT and pMCT, were confirmed by restriction enzyme digestion and DNA sequencing. To make the CAT reporter constructs, an approximately 1631 bp fragment containing the cat-SV40 polyA sequences was isolated by digestion of pU3-R-CAT with *Hind*III and *Bam*HI. The cat-SV40 polyA fragment was gel-purified and the ends were made blunt using T4 polymerase. An *Asp*718 linker [5'-GCTAGC GGTACC-3' (SEQ ID NO:9)] was ligated to the blunt ends and the fragment was cloned into *Asp*718 digested pSP72-3'LTR, pMT or pMCT to generate pSP72-3'LTRcat, pMT-cat and pMCT-cat, respectively.

The entire sequence of the recombinant M-MuLV LTR present in pMT-cat is:

5'-AATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGC
AAGGCATGGAAAAATACATAACTGAGAATAGAGAAGTTCAGATCAAGGTCAGGAA
CAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGC
CCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATC
TGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGC
GGTCCAGCCCTCAGCAGTTTCTAGAGTACTTCAAGAACTGCTGACATCGAGCTTGCT
ACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGG
GAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTC
TCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACT
GCTTAAGCCTCGAATTCAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGGGCGCC
AGTCCTCCGATTGACTGAGTCGCCCGGGTACCCGTGTATCCAATAAACCCTCTTGCA
GTTGCATCCGACTTGTGGTCTCGCTGTTCCTTGGGAGGGTCTCCTCTGAGTGATTGAC
TACCCGTCAGCGGGGGTCTTTCATT-3' (SEQ ID NO:16).

The entire sequence of the recombinant M-MuLV LTR present in pMCT-cat is:

5'-AATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGC
AAGGCATGGAAAAATACATAACTGAGAATAGAGAAGTTCAGATCAAGGTCAGGAA
CAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGC
CCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATC
TGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGC
GGTCCAGCCCTCAGCAGTTTCTAGCTGGAGTTCCGCGTTACATAACTTACGGTAAAT
GGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTAT

GTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGAGTTTGTTTTG
GCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCA
AATGGGCGGTAGGCGTGTACTCTAGATGCTACATATAAGCAGCTGCTTTTTGCCTGT
ACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGG
AACCCACTGCTTAAGCCTCGAATTCAGCTCAATAAAAGAGCCCACAACCCCTCACTC
GGGGCGCCAGTCCTCCGATTGACTGAGTCGCCCGGGTACCCGTGTATCCAATAAACC
CTCTTGCAGTTGCATCCGACTTGTGGTCTCGCTGTTCCTTGGGAGGGTCTCCTCTGAG
TGATTGACTACCCGTCAGCGGGGGTCTTTCATT-3' (SEQ ID NO:17).

The promoter activities of pMT-cat and pMCT-cat were compared with a wild type M-MuLV LTR, the HIV-1 LTR and the CMV-IE promoter in the presence or absence of HIV-1 Tat in DNA transfection experiments as described below.

EXAMPLE 4

The Modified M-MuLV LTR Promoter Functions Efficiently In A Wide Variety Of Human Cell Types And Is Trans-Activated By Tat

Promoter activities of the modified M-MuLV LTRs in the presence or absence of the HIV-1 Tat protein were tested by transfecting a series of CAT reporter constructs into a panel of human cell lines.

A. Expression Of The pMCT And pMT Promoters In A Panel Of Human Cell Lines

The promoter activities of the modified M-MuLV LTRs (pMT-cat and pMCT-cat constructs) were compared with the wild type M-MuLV LTR, the HIV-1 LTR and the CMV-IE promoter in a variety of human cell lines. The ability of the promoters to be trans-activated by the Tat protein was also examined.

The following cell lines were used. HeLa, a human epithelioid carcinoma (ATCC CCL 2); HepG2, a human hepatoma line (ATCC HB 8065); HuH-7, a human hepatoma cell line [Nakabayashi, H. *et al.* (1982) Cancer Res. 42:3858]; CCRF-CEM (CEM), a human lymphoblastic cell line (ATCC CCL 119); and H9, a human T-cell lymphoma (ATCC HTB 176).

The following plasmids were used. pMLV LTR-cat (wild type M-MuLV LTR), pMT-cat (modified M-MuLV LTR containing the TAR element), pMCT-cat (modified M-MuLV

LTR containing the CMV-IE promoter and the TAR element), pHIV LTR-cat (wild type HIV LTR) and pCMV-cat (CMV-IE promoter). The Tat expressing plasmid, pCEPtat, was co-transfected with each reporter plasmid to examine the ability of the Tat protein to trans-activate a given promoter. The ratio of reporter plasmid DNA to Tat plasmid DNA was 10:1. Plasmid DNA was prepared as described in Example 1.

Plasmid DNA was introduced by electroporation into the suspension lines, CEM and H9, and by calcium phosphate precipitation into the monolayer cell lines, HeLa, HepG2 and HuH-7.

Electroporations were performed as follows. Prior to electroporation, the CEM and H9 cells were grown in RMPI 1640 medium (Gibco-BRL) containing 10% fetal bovine serum (FBS, Gibco-BRL) and penicillin and streptomycin in an atmosphere containing 5% CO_2 at 37°C. Approximately 10 x 10^6 cells (0.4 ml) were placed in a 0.4 cm cuvette (BioRad), and plasmid DNA containing the desired CAT reporter gene (10 µg) was added. When the Tat protein was to be expressed 10 µg of reporter plasmid and 1 µg of Tat plasmid were used in the electroporation. The cells were then electroporated using a Gene Pulser (BioRad) at 960 µF and 300 V (with a time constant of 7.5-11 msec). The cells were then transferred into a T25 flask (Falcon) containing 10 ml of RPMI-1640 medium containing 20% FBS.

Following a 48 hr incubation, the cells were centrifuged at 600 X *g* for 5 minutes and then washed once in cold phosphate buffered saline. The cell pellet was resuspended in 100 µl Tris (25 mM, pH 7.8), and then freeze-thawed three times using a 37°C water bath and a dry-ice bath. The cell pellet was vortexed after each thaw. The lysate was recovered by centrifugation at full speed (approximately 14,000 rpm) in a microcentrifuge (Brinkmann, Model 5415C) for 2 minutes.

The HeLa, HepG2 and HuH-7 cell lines were transfected using the calcium phosphate precipitation method described in Example 1. The transfected cells were harvested 48 hours after the addition of DNA. Cell lysates were prepared as described in Example 1.

Cell lysates were assayed for CAT activity as follows. Approximately 20 µl of the lysate was mixed with 75 µl Tris (1 M, pH 7.8), 5 µl acetyl coenzyme A (3.5 mg/ml) and 3 µl of ^{14}C-chloramphenicol. This reaction mixture was incubated at 37°C for 45 minutes, then vortexed with 1 ml ethyl acetate. The top layer was transferred to a 1.5 ml microcentrifuge tube (Eppendorf) and dried under vacuum for 1 hour. The dried product was resuspended in 30 µl ethyl acetate, spotted onto a TLC plate (Whatman) and developed with 95% chloroform

and 5% methanol in a closed glass tank. The developed TLC plate was analyzed by autoradiography or using a phosphoimager as described in Example 1.

The efficiency of the transfections was controlled for by co-transfecting an internal control plasmid (human growth hormone). Levels of CAT activity were normalized to this internal control. Each transfection included 0.1 µg of pXGH5 plasmid (Nichols Institute Diagnostics) which expresses human growth hormone into the culture supernatant. Quantitation of the human growth hormone was done using the ELISA kit provided by Nichols Institute Diagnostics.

Table 1 summarizes the results of these transfection experiments. The relative level of CAT expression is shown for each construct in the absence (-Tat) or the presence of Tat (+Tat).

TABLE 1

Relative Levels Of CAT Expression (-Tat / +Tat)[a]

Cells:	pMLV LTR-cat	pMT-cat	pMCT-cat	pHIV LTR-cat	pCMV-cat
HeLa	3.3 / 4.0	3.5 / 2.2	9.0 / 36.3	1.0 / 34.7	45.2 / 19.9
HepG2	0.6 / 0.5	0.2 / 0.2	5.4 / 11.5	1.0 / 10.7	12.1 / 14.2
HuH-7	0.7 / 1.0	0.4 / 0.5	7.7 / 19.0	1.0 / 22.0	18.6 / -
CEM	24.0/ 28.0	2.9 / 10.0	2.2 / 35.0	1.0 / 30.0	52.8 / 48.1
H9	13.0 / -	0.2 / 0.2	5.6 / 63.0	1.0 / 42.0	43.0 / -

[a] For each cell type, results are reported relative to the level of CAT activity generated by pHIV LTR-cat in the absence of Tat (this level is arbitrarily assigned the value of 1.0). The CAT activities represent reproducible mean values from at least three independent experiments and were normalized to the expression of a human growth hormone plasmid construct as described.

[b] "-", undetermined.

As shown in Table 1, in the absence of Tat, the pMCT promoter (pMCT-cat) exhibited activities 2-5 fold lower than the CMV-IE promoter (pCMV-cat), but considerably higher than the wild type M-MuLV LTR (pMLV LTR-cat). However in the presence of Tat, the pMCT-cat construct generally exhibited equal or higher activity than did pCMV-cat. On the other hand, the pMT-cat construct exhibited poor activity which was similar to pMLV LTR-cat. Furthermore, pMT-cat was not responsive to Tat, despite the fact that it contains the HIV-1 TAR element. This experiment shows that the modified M-MuLV LTR present in pMCT is a strong promoter in both hepatoma cells and T lymphocytes, whereas the wild type M-MuLV LTR is moderately active only in lymphocytes. These results also demonstrate that the enhancer element of the CMV-IE gene is essential to permit Tat trans-activation (pMT lacks this CMV element and is not capable of responding to Tat).

B. Expression Of The pMCT Promoter In A Human B Lymphoblastoid Cell Line

As shown above, promoter activity clearly varies depending upon the cell type. To characterize the pMT and pMCT promoters further, the activity of these promoters was examined in the human B-lymphoblastoid cell line AA2 [Chaffee, S. *et al.*, J. Exp. Med. 168:605 (1988); AA2 cells are available from the AIDS Research and Reference Reagent Program, NIH, Bethseda, MD, catalog no. 135]. In these experiments, a cellular β-actin promoter construct [pβactin-cat; Ng, S.-Y. *et al.* (1985) Mol. Cell. Biol. 5:2720] as well as the CMV-IE promoter [pCMV-cat; Hunninghake, G.W. et al. (1989) J. Virol. 63:3026] were included for comparison. All promoters were assayed either in the presence or absence of Tat.

Plasmid DNA was introduced into AA2 cells by electroporation as described above with the exception that AA2 cells were grown in RMPI 1640 medium (Gibco-BRL) containing 10% fetal bovine serum (FBS, Gibco-BRL), 1X non-essential amino acids (Gibco-BRL) and 1 mM pyruvate (Gibco-BRL) and penicillin (50 units/ml; Gibco-BRL) and streptomycin (50 µg/ml; Gibco-BRL) in an atmosphere containing 5% CO_2 at 37°C. The AA2 cells were electroporated at 250 µF and 300 V (with a time constant of 7.5-11 msec). Cell lysates were prepared 48 hours after the addition of DNA. Cell lysates were prepared and assayed for CAT activity as described above.

Figure 11 shows the results of these transfection experiments. As shown in lane 7 of Figure 11, the M-MuLV-CMV-TAR (pMCT) construct exhibited high promoter activity in

the absence of Tat. As shown in lane 8 of Figure 11, in the presence of Tat, the pMCT promoter was trans-activated significantly. Thus, in AA2 cells, the pMCT promoter gave the highest level of expression among the promoters examined, whether or not Tat was present. It is possible that the high levels of CAT expression induced by pMCT were due to the existence of a heterologous viral trans--activator in AA2 cells (the AA2 cell was established by EBV-transformation). The pMCT construct may have the added advantage that it expresses at higher levels in cells which are infected with or transformed by a number of different viruses (*i.e.*, in addition to HIV).

In contrast, the M-MuLV-TAR (pMT) construct showed low basal activity and was not responsive to Tat (see lanes 1 and 2 of Figure 11). In comparison to the strong CMV-IE promoter, the M-MuLV-CMV-TAR (pMCT) promoter exhibited 2-fold higher basal activity (*i.e.*, in the absence of Tat). The M-MuLV-CMV-TAR (pMCT) promoter was responsive to Tat as shown by the 4-5 fold increase in activity (relative to the basal activity) seen in the presence of Tat.

These results indicate that the M-MuLV LTR and HIV TAR combination (present in pMT) is not sufficient to confer Tat-responsiveness upon the M-MuLV LTR. In contrast the CMV-TAR modification (present in pMCT-tat) allows for the transactivation of the pMCT promoter and further provides a hybrid promoter having a high basal level of activity in human cells.

C. Expression Of The Modified M-MuLV LTR In A Human Cell Line Constitutively Expressing Tat

Expression levels from pMCT-cat in the presence of Tat were further assessed using a human T lymphoma cell line, CEM-TART [Chen, H. *et al.*(1992) Proc. Natl. Acad. Sci. USA 89:7678; CEM-TART cells are available from the AIDS Research and Reference Reagent Program, NIH, Bethseda, MD]. CEM-TART cells constitutively express the HIV-1 Tat protein. The following CAT reporter constructs were electroporated into CEM-TART cells: pCMV-cat, pLLL-cat (contains the wild type MuLV LTR), pMCT-cat and pHIV LTR cat.

CEM-TART cells were grown and electroporated as described above for CEM and H9 cells in Example 4A. Plasmid DNA was purified as described in Example 1. Cell lysates were prepared 48 hours after the addition of the DNA and CAT assays were performed as described above in Example 1.

Figure 12 shows the relative levels of CAT expression in CEM-TART cells. These results indicate that the pMCT promoter exhibited the highest activity among M-MuLV LTR, CMV-IE, and HIV-1 LTR promoters. Therefore, for the purpose of targeting HIV-1 infected cells, the pMCT construct appears to be ideal, since it exhibits high activity in the absence of Tat, and is strongly activated in the presence of Tat (*i.e.*, during HIV-1 infection).

EXAMPLE 5

Transcription Initiates From Both TATA Boxes In The pMCT LTR Construct

The pMCT LTR construct contains both the M-MuLV TATA box and the HIV-1 TATA box. To determine whether transcription initiated from one or both of these TATA boxes the following experiments were performed. HepG2 cells were transfected with pMCT-cat plasmid and the RNA transcripts were analyzed by Northern blot analysis and RNase mapping.

HepG2 cells were transfected with plasmid DNA [10 μg/T25 flask (Falcon)] in the presence or absence of pCEPtat (2 μg/T25 flask) by the calcium phosphate method as described in Example 1 except that all reagents were scaled up 2.5 times. Cells were washed with cold PBS two times 24 hr after DNA removal and scraped into 1 ml of PBS in a 1.5 ml microcentrifuge tube. The cells were pelleted by centrifugation for 1 min at 3000 rpm. The cells were resuspended in 250 μl of Solution I (10 mM Tris, pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, RNase-free) containing 25 μl of VRC (BRL) and incubated on ice for 5 min. Twelve and one-half microliters of 10% NP40 was added and the tube was vortexed briefly and centrifuged in a microcentrifuge at 4000 rpm for 3 min. 250 μl of the supernatant was transferred into a second tube containing 250 μl of 2 X proteinase K buffer (200 mM Tris, pH 7.5, 40 mM EDTA and 300 mM NaCl), and 25 μl of 10% SDS, 10 μl of 5 mg/ml proteinase K were added. The solution was incubated at 37°C for 30 min to 1 hr. To isolate the polyA$^-$ RNA, the solution was brought to 0.5 M NaCl, 1% SDS and approximately 30 μl of oligo-dT cellulose powder (CRI) was added and the tube was rotated at room temperature for 1 hr. The oligo-dT cellulose was pelleted and washed twice with 650 μl of high-TEN-SDS (20 mM Tris, pH 7.5, 10 mM EDTA and 0.5 M NaCl, 1% SDS) and once with 650 μl of low TEN-SDS (as above but using 0.1 M NaCl instead of 0.5 M NaCl). The RNA was eluted with 200 μl TE (10 mM Tris, pH 7.5, 1 mM EDTA) twice and precipitated with 40 μl of 5 M ammonium acetate and 1 ml of 95% ethanol.

For Northern blot analysis, polyA$^+$ RNA isolated from HepG2 cells transfected with pMCT-cat in the presence or absence of pCEPtat as described above. The RNA was electrophoresed on a 1.6% formaldehyde agarose gel. The RNA was transferred to a nylon membrane [Genescreen (DuPont)] and probed using the cat gene present in pMCT-cat. pMCT-cat was radiolabeled using the Prime-a-Gene labelling system (Promega). In order to control for the amount of RNA loaded in each lane, the blot was stripped of the cat probe and rehybridized with a β-actin probe [pβ-actin: Karlsson, R. *et al.* (1991) Mol. Cell. Biol. 11:213].

Figure 13 shows the autoradiograph of the Northern blot. The radioactivity on the membrane (*i.e.*, the Northern blot) was quantitated using a phosphoimager (Fuji Bio-imaging analyzer BAS 2000). Comparison of lane 1 (pMCT-cat alone) with lane two (pMCT-cat plus pCEPtat) shows that Tat trans-activated synthesis of CAT RNA from the pMCT promoter up to 16 fold.

For RNase mapping, the probe was made using pU3CMV5'CAT. pU3CMV5'CAT was constructed by inserting the *Nhe*I to *Eco*RI fragment (approximately 930 bp) of pMCT-cat (Example 3) into pSP72 (Promega) digested with *Xba*I and *Nhe*I. pU3CMV5'CAT was digested with *Sal*I and 0.5 µg of linear DNA was transcribed with phage T7 polymerase (Promega) using the according to the manufacturer's protocol. The reaction contained 4 µl of 5x *in vitro* transcription buffer (Promega), 2 µl of 0.1 M DTT, 0.5 µl of RNasin (20 units), 1 µl each of 10 mM ATP, GTP and CTP, 2 µl of 100 µM UTP, 1 µl of linear DNA (0.5 µg), 5 µl (50 µCi) of α^{32}P-UTP (DuPont NEN catalog # NEG-007H), 5.5 µl of ddH_2O and 1 µl of T7 polymerase (10 units) and incubated at 37°C for 1 hr. The DNA template was digested with 1 µl of RNase-free DNase (1 unit/µl) at 37°C for 10 min in the presence of 20 µg of yeast tRNA (in 2 µl). The labeled RNA was precipitated with 80 µl of TE, 20 µl of 5 M ammonium acetate, and 360 µl of ethanol. After pelleting, the RNA was resuspended in 50 µl of formamide and stored at -20°C until used.

RNase mapping was performed by mixing 25% of the polyA$^+$ RNA isolated from the transfected HepG2 cells with 2 µl of the RNA probe in 30 µl of 1 X hybridization buffer containing 80% formamide (5X hybridization buffer comprises: 2 M NaCl, 5 mMEDTA and 0.2 M MOPS, pH 7.0) at 90°C for 5 min. The temperature was then decreased to 40°C overnight. To the hybridization solution, 300 µl of RNase digestion buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 0.3 M NaCl, 40 µg/ml RNase A, 2 µg/ml RNase T1) was added and the tube was incubated at 30°C for 1 hr. After RNase digestion, 10 µl of 20% SDS and 5 µl of

proteinase K (10 mg/ml) were added and the tube was incubated at 37°C for 15 min. The final product was extracted with phenol-chloroform followed by extraction with chloroform and precipitated with 20 μg of yeast tRNA carrier by adding 1 ml cold ethanol and incubation at 70°C for at least 3 hr.

The RNA-RNA hybrids were resolved on a 5% neutral polyacrylamide gel. *Bst*EII-digested lambda DNA (BRL) was run on the gel to provide a marker. The gel was dried and exposed to X-ray film and quantified using a phosphoimager (Fuji) as described in Example 1.

Figure 14 shows the results of the RNase mapping. Figure 15 is a schematic showing the probe and the location of the TATA boxes in pMCT. If both transcription initiation sites are used two species of RNA will be protected generating bands of 434 and 314 nucleotides in length. The data show that both the HIV and the M-MuLV transcription initiation sites were used in HepG2 cells transfected with the pMCT-cat plasmid (Figure 12B). The upstream HIV initiation site was preferred 8-10 fold over the downstream M-MuLV site (Figure 12B). Tat up-regulated the synthesis of both species of RNA (17-fold from the HIV promoter and 22-fold from the M-MuLV promoter) suggesting that although TAR is not present on the M-MuLV transcript, the rate of transcription from the downstream M-MuLV transcription unit was still mediated by upstream Tat-TAR interaction.

The results shown in this Example demonstrate that the LTR present in pMCT is inducible by the Tat protein. The amount of RNA produced from the pMCT promoter increased 16 to 22 fold over basal levels when Tat was present (16 fold as judged by Northern blot analysis; 17 to 22-fold as judged by RNase mapping).

EXAMPLE 6

Generation Of Improved Retroviral Vectors Containing Modified 3' LTRs With Or Without Extended Packaging And Splicing Signals

pMT and pMCT are single LTR plasmids. In order to generate a retroviral vector containing these improved promoters, the construct must contain both a 5' and a 3' LTR. Retroviral vectors containing these modified LTRs were constructed.

In order to increase the level of expression of genes inserted into retroviral vectors, the amphotropic M-MuLV vector, pLNL6, was modified. The pLNL6 vector was used as the

starting point for the creation of improved retroviral vectors because pLNL6 has been approved for use in clinical therapy protocols.

Specifically, the M-MuLV LTR was reconstructed to produce a retroviral vector containing the CMV-IE enhancer and a HIV-1 trans-activation response (TAR) element (the pMCT promoter). This novel recombinant M-MuLV LTR contains MLV and CMV enhancer elements, two TATA promoters (from HIV and MLV) and the HIV-1 TAR element. These modifications were carefully designed so that important M-MuLV functions such as reverse transcription, packaging and polyadenylation of viral RNA would not be disrupted. The CMV-IE enhancer was chosen as it functions as a strong enhancer in a wide variety of cell types. The TAR element directs very high levels of expression in cells which are expressing the HIV-1 trans-activator Tat (*i.e.*, HIV-1 infected cells). The TAR element also competes for the HIV-1 protein Tat which induces the production of HIV-1 in the infected cells. These features result in high levels of anti-HIV genes to be expressed and prevent the spread of the HIV virus in the body's immune system, both by limiting production of HIV from cells already infected, and by allowing the immune system to be gradually re-populated with immune cells which can no longer be infected. The combination of the CMV-IE enhancer and TAR increases the level of expression of inserted genes in HIV infected cells. Additionally, because the LTR of pLCTSN contains a very strong promoter, this vector is ideal for expression of inserted genes in a wide variety of mammalian cell types (including non-HIV infected cells). The ability to express a gene to high levels in mammalian cells will facilitate studies of gene expression, lineage mapping studies, etc.

This improved retroviral vector (pLCTSN) was further modified to include extended packaging and splicing signals from the M-MuLV genome (creating pLGCTSN). These modifications were designed to increase the efficiency of packaging the vector RNA into viral particles.

Figure 16 depicts schematically these modified MuMLV vectors. Modifications to generate vectors containing LTRs with improved promoter function were made only to the 3' (M-MuLV) LTR. These modifications will appear in both LTRs following infection, reverse transcription and integration of the vector sequences. The designation "(cat)" used in Figure 16 indicates that the CAT gene may be inserted into the polylinker located in the pLLL vector or the polylinker located upstream of the SV-neo gene on the pLSN, pLCTSN and pLGCTSN vectors. The polylinker may also be used for the insertion of any desired sequence such as sequences comprising anti-HIV genes.

a) Construction Of pLLL

pLLL was constructed using pLNL6 (SEQ ID NO:1) as a starting point. pLNL6 contains the M-MuLV promoter in the 3' LTR and the murine sarcoma virus (MSV) promoter in the 5' LTR. For ease in subsequent cloning steps, the few cloning sites and the internal SV-neo gene present in pLNL6 were removed and replaced with a synthetic polylinker to generate pLLL (shown schematically in Figure 16).

To construct pLLL, 1 µg of pLNL6 was digested with 5 units of *Cla*I in NEB buffer #4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT at pH 7.9), in a final volume of 20 µl at 37°C for 1 hour. Then, 5 units (0.5 µl) of *Bcl*I were added and the reaction temperature was increased to 50°C for 1 hour. To isolate the vector fragment, the reaction mixture was mixed with 2 µl of glycerol-dye (10% glycerol, 1 mM EDTA, 0.1% xylene cyanol FF, and 0.1% bromphenol blue) and electrophoresed on a 1% agarose gel until the dye front had move two thirds down the length of the gel. The upper band was visualized with a hand-held UV box (UVP Model UVGL-25, 366 nm) and cut out with a razor blade. This agar block was transferred to a 1.5 ml microcentrifuge tube (Eppendorf) and the DNA was isolated using GeneClean (Bio-101) according to the manufacturer's instructions. The volume of the agar block containing the *Cla*I digested vector was measured. Sodium iodide solution was added to the tube containing the agar block at a volume 3-5 times that of the agar block. The agar was melted by incubation at 60°C for 5-10 minutes. To this mixture, 1 µl of glass milk (glass milk was provided in the GeneClean kit from Bio-101) was added and the mixture was incubated at room temperature for 5 min. The tube was centrifuged briefly in a microcentrifuge (Brinkmann) at 14,000 rpm for 10 sec. The pellet was washed twice with 600 µl of GeneClean wash at -20° C. The DNA was then eluted twice using 10 µl of ddH_2O at 60°C.

The double stranded insert containing the polylinker site was constructed using the following two oligonucleotides: 5'-GATCTAAGCTTGCGGCCGCAGATCTCGAGCCATGG ATCCTAGGCCTGATCACGCGTCGACTCGCGAT-3' (SEQ ID NO:2) and 5'-CGATCGCG AGTCGACGCGTGATCAGGCCTAGGATCCATGGCTCGAGATCTGCGGCCGCAAGC TTA-3' (SEQ ID NO:3).

These oligonucleotides were mixed together in 20 µl ddH_2O, heated at 85°C for 5 minutes and gradually cooled down to room temperature over a 1 hour period. To this tube, 2.3 µl of 10X kinase buffer (700 mM Tris-HCl, 100 mM $MgCl_2$ and 50 mM DTT, pH 7.6), and 1 µl of T4 polynucleotide kinase (NEB) were added. The mixture was incubated at 37°C

for 1 hour. The kinase activity was then inactivated by incubating the mixture at 65°C for 1 hour.

Ligation of the vector and the oligonucleotide insert was performed in 10 µl of a reaction mixture comprising 1X ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 25 µg/ml bovine serum albumin, and 26 µM NAD+, and pH 7.8), 0.05 µg of the pLNL6 vector, 0.01 µg of insert, and 0.5 unit of T4 DNA ligase (IBI) at 15°C overnight. The ligation mixture was used to transform competent DH5α cells (BRL) as follows. Two µl of the ligation mixture was added to 20 µl of competent cells and incubated at 4°C for 30 min. The cells were then subjected to a temperature shock by incubation at 40°C for 1 min. and placed in a 37°C shaker (250 rpm) for 1 hour before being plated onto an ampicillin agar plate (LB plus 1.5% agar and 0.1 mg/ml ampicillin) and incubated in a 37°C incubator overnight. On the second day of incubation, colonies (usually 12) were picked from the ampicillin agar plate and placed in 3 ml superbroth with ampicillin (100 µg/ml). The tube was incubated overnight at 37°C with shaking.

Plasmid DNA was prepared by the boiling method [Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 1.34-1.35]. Approximately 1.4 ml of the overnight culture was transferred into a 1.5 ml microcentrifuge tube (Eppendorf) and the bacteria were pelleted by centrifugation in a microcentrifuge for 2 min and the supernatant was removed. The pellet was resuspended completely in 50 µl STET buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0) by vortexing. The bacteria were lysed by the addition of 4 µl of 5-50 mg/ml lysozyme solution (Sigma, frozen stock stored at -20°C) and the tube was boiled for exactly 1 min in a boiling water bath. The lysate was centrifuged for 10 min in a microcentrifuge, and the scum pellet was discarded with a toothpick. To the supernatant an equal volume (50 µl) of cold isopropanol (-20°C) was added, the tube was mixed and incubated at -20°C for 10 min to precipitate plasmid DNA. The plasmid DNA was pelleted for 5-10 min in a microcentrifuge and the pellet was resuspended in 50 µl of ddH$_2$O. For restriction enzyme mapping, 10 µl of the DNA was digested with the appropriate restriction enzymes in a final reaction volume of 20 µl.

The plasmid DNA was then mapped by *Sst*I, *Sst*I/*Hin*dIII, and *Asp*718/*Bam*HI digestion. The clone containing the sequence of interest was grown in a large preparation as described in Example 1.

The site of insertion was confirmed by DNA sequencing using the following primers 5'-GAACCTCCTCGTTCGACC-3' (SEQ ID NO:18), and 5'-AACTAGAGCCTGGACCAC-3' (SEQ ID NO:19). These primers contain sequences which correspond to sequences located 5' and 3' to the insertion. The sequencing reagents and methods used were those provided by USB in the Sequenase kit. Briefly, 5 µl of plasmid (containing about 4 µg DNA) was mixed with 1 µl of primer (10 ng), 1 µl of 1 N NaOH and incubated at 37°C for 10 min. To the mixture, 1 µl of 1 N HCl and 2 µl of 5 X Reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) were added and incubated at 37°C for 5 min. To the tube, the following reagents were added in order: 1 µl 0.1 M DTT, 2 µl diluted labeling mix (diluted 5 to 1 dilution of 7.5 µM dGTP, 7.5 µM dCTP, and 7.5 µM dTTP), 0.5 µl ^{35}S-dATP (Amersham), 2 µl diluted Sequenase (diluted 8 to 1 in enzyme dilution buffer: 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA and 50% glycerol), and the reaction was incubated at room temperature for less than 5 min. To a 96-flex well plate (Falcon) marked with A, T, C, G, 2.5 µl of ddNTP termination mix (ddA: 80 µM dNTP, 8 µM ddATP, 50 mM NaCl; ddT: 80 µM dNTP, 8 µM ddTTP, 50 mM NaCl; ddC: 80 µM dNTP, 8 µM ddCTP, 50 mM NaCl; ddG: 80 µM dNTP, 8 µM ddGTP, 50 mM NaCl) was added to the designated well on the side of the wall and the plate was incubated at 37°C for 1 min. To the bottom of the flex well plate, 3.5 µl of the Sequenase reaction mix was added, and the reaction was started by hitting the plate gently to mix all the reagents simultaneously. The reaction was incubated at 37°C for 5 min. To stop the reaction, 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% of bromphenol blue and 0.05% xylene cyanol FF) was added to each well, on the side, and hit to stop all reactions simultaneously. The 96-well was heated to 75°C for 2 min before loading onto a sequencing gel. The gel was electrophoresed at 2000 volt for a period of time depending on the length of the readable sequences. The gel was transferred to a piece of 3 MM paper (Whatman) and dried under vacuum. The sequence was determined by exposing the dried gel to a XAR5-OMAT film (Eastman Kodak Co.).

pLLL is shown schematically in Figure 16. DH5α bacterial cells harboring pLLL were deposited with the American Type Culture Collection; pLLL was assigned ATCC No. ___.

pLLL was then used to construct a series of two-LTR retroviral vectors: pLSN, pLCTSN and pLGCTSN. These vectors were further modified by the insertion of a CAT reporter gene. The resulting vectors may be referred to as pLSN-cat, pLCTSN-cat and pLGCTSN-cat, respectively. The CAT gene was isolated from pCAT3M as a *Bgl*II/*Sau*3AI fragment: this fragment was cloned into the *Bam*HI site located in the polylinker region

located upstream of the SV-neo gene in each of the above plasmids. CAT gene sequences are available from a variety of commercial sources including pCAT-Control vector (Promega). pLSN, pLCTSN and pLGCTSN all contain a SV40 promoter driving the selectable marker *neo* (shown schematically in Figure 16 and referred to as the SV-neo gene).

b) Construction Of pLSN

To generate a vector containing a selectable marker which allows for the isolation of cells which have incorporated the vector DNA, pLSN was created. pLSN contains the *neo* gene under the transcriptional control of the SV40 enhancer/promoter; pLSN may also contain the CAT reporter gene (shown schematically in Figure 16); the CAT gene may be inserted into the polylinker region upstream of the SV-neo gene in pLSN. pLSN functions as the wild type vector control in subsequent transfection experiments.

To create pLSN, a *Bam*HI/*Stu*I fragment containing SV40 enhancer/promoter was isolated from pLNSX [Miller, A.D.and Rosman, G.J. (1989) BioTechniques 7:980]. pLNSX and pLLL were digested with *Bam*HI and *Stu*I in NEB buffer #2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). The digestion products were purified by agarose gel electrophoresis as described above for the construction of pLLL. A small fragment of approximately 350 bp which contained the SV40 promoter from pLNSX was cloned into the pLLL vector. The final product, designated pLLL/SV40, was confirmed by restriction enzyme digestion using *Bam*HI and *Cla*I.

In order to insert a better translation initiation codon at the beginning of the *neo* gene, the *neo* gene was isolated from pLNSX using PCR. *Pfu* polymerase (Stratagene) was used to amplify the gene. This amplification was conducted in 5 µl of 10x *Pfu* reaction buffer, 0.5 µl of dNTP (15 mM), 0.5 mM of each of the following primers: 5'-AAGCTTGATCACCACCA TGATTGAACAAGATGG-3' (SEQ ID NO:4) and 5'-CCGGATCCGTCGACCCCAGAGTCC CGCTCAGAAG-3' (SEQ ID NO:5), 0.5 µl of pLNSX (0.01 µg) and 38 µl of ddH_2O. These primers contain the modified translation initiation control sequence (-CCACCATG-), as this modification was found to greatly increase the strength of the *neo* gene in tissue culture cells [Kozak, M. (1986) Cell 44:283].

The mixture was heated at 95°C for 5 minutes and 1 µl of *Pfu* polymerase was added. This reaction mixture was cycled through 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes. After amplification, the DNA was precipitated with a 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol and then separated on a 1% agarose gel, as

described above for the construction of pLLL. Purification of the DNA band from the agarose gel, ligation of the fragment with the *Bcl*I-digested pLLL/SV40 vector, screening, purification and confirming of plasmid pLSN was conducted as described above.

c) Construction Of pLCTSN

pLCTSN contains the modified M-MuLV LTR present in the pMCT construct. pLCTSN was generated by the following four-fragment ligation. The vector pLSN was digested with *Sac*II and *Kpn*I. The three inserts were *Sac*II to *Xho*I of pLSN, *Xho*I to *Nhe*I of pLSN, and *Nhe*I to *Kpn*I of pMCT. Isolation of DNA fragments, ligation, transformation of competent DH5a, screening of colonies, and mapping of the positive clones were performed as described above for the construction of pLSN.

pLCTSN is shown schematically in Figure 16. DH5α bacterial cells harboring pLCTSN were deposited with the American Type Culture Collection: pLCTSN was assigned ATCC No. ___.

d) Construction Of pLGCTSN

pLGCTSN contains the modified M-MuLV LTR present in the pMCT construct and contains extended packaging signals and a 3' splice acceptor site from the M-MuLV genome. The addition of these sequences were made to improve the packaging efficiency of the vector RNA and to avoid interference by the native 5' splice donor site. The 5' splice donor site does not have a corresponding splice acceptor in the pLCTSN vector. This modification stabilized transcripts expressed by the vector and enhanced the packaging efficiency of vector genomes.

pLGCTSN was cloned by inserting an *Spe*I-*Bam*HI fragment of pDGLtax/rex [Akagi, T. *et al. Gene* 106: 255 (1991)] into the same sites of pLCTSN. This *Spe*I/*Bam*HI fragment contains the extended packaging signal and a 3' splice acceptor site from the HIV-1 genome. The positive clones were confirmed with *Spe*I, *Bam*HI, *Kpn*I and *Bgl*II digestions. pLGCTSN is shown schematically in Figure 16.

DH5α bacterial cells harboring pLGCTSN were deposited with the American Type Culture Collection: pLGCTSN was assigned ATCC No. ___.

EXAMPLE 7

Packaging Efficiency Of The Improved M-MuLV Vectors

In addition to providing more efficient promoters, increasing the vector titer of the packaging cells is yet another key issue in retroviral gene therapy and a goal of the invention. The modifications made to create the new retroviral vectors pLSN, pLCTSN or pLGCTSN were examined to determine the effect upon the packaging efficiency of vector sequences. The packaging cell line, PA317 [Miller, A.D. and Buttimore, C. (1986) Mol. Cell. Biol. 6:2895 and Miller, A.D. (1990) Hum. Gene Ther. 1:5], was transfected with pLSN, pLCTSN or pLGCTSN using lipofectamine (BRL). Lipofection was carried out according to the manufacturer's protocol.

PA317 cells were grown in DMEM containing 10% FBS and penicillin and streptomycin in an atmosphere containing 10% CO_2 at 37°C. Twenty hours prior to lipofection, PA317 cells were placed into a T25 flask (Falcon) at 50% confluency (approximately 1 x 10^6 cells/flask). To transfect the cells, DNA (4 µg) was added to 300 µl serum-free DMEM not containing antibiotics in a microcentrifuge tube (Eppendorf), and mixed gently. In a 15 ml polycarbonate tube (Falcon), 300 µl serum-free DMEM and 12 µl of lipofectamine were mixed gently. The two solutions were combined by adding the DNA-containing solution dropwise into the lipofectamine tube, and the mixture was incubated at RT for 45 min. Following this incubation, 2 ml of serum-free DMEM was added and mixed gently. The cells were washed with serum-free DMEM and the DNA/lipofectamine mixture was gently added to the cells. The cells were incubated at 37°C in a 10% CO_2 incubator for 5 hr. After the 5 hr incubation, 2.5 ml of DMEM containing 20% FBS and antibiotics was added to the T25 flask and the cells were incubated overnight. Twenty hours after the 5 hr incubation, the medium was replaced with fresh DMEM containing 20% FBS and antibiotics. For vector titration, the medium was changed at 24 hr after the medium was replaced with fresh DMEM and virus was harvested 24 hr later. When cells were to be cloned, the transfected PA317 cells were split at a 1:10 ratio into the appropriate selective medium.

Packaging efficiency was determined by infecting HeLa and HuH-7 cells using the virus stocks generated from transfected or selected PA317 cells. Duplicate plates of PA317 cells were transiently transfected with equimolar amounts of the retroviral vector DNAs using the lipofection protocol above. Supernatants were harvested 24 hr after transfection and assayed for the presence of viral particles. The recombinant viruses were titered by retroviral

transduction of HeLa and HuH-7 cells and quantitation of the resulting G418-resistant colonies.

Infections were carried out as follows. HeLa and HuH-7 cells were split into 6-well plates at a density of 5 x 10^5 cells per well 17-20 hr prior to infection. Cells were infected with virus stock (prepared as described above) at dilutions of 1:100, 1:1000 and 1:10,000 in 500 µl of growth medium (DMEM containing 10% FBS and antibiotics) containing 4 µg/ml polybrene (Sigma) for 2 hr. The plates were fed with 2 ml of growth media. Twenty-four hr after adding the growth medium, the cells were split at a ratio of 1:20 into selective medium [DMEM containing 10% FBS, penicillin, streptomycin and 5 mg/ml G418 (Geneticin™, BRL)]. G418-resistant colonies were counted about 8-10 days later by coomassie brilliant blue G staining (1 g/ liter in 40% methanol and 10% acetic acid; Miller, A.D. et al. (1993) Methods in Enzymology 217:581). The results were calculated based on 2-3 sets of repeats.

Table 2 shows the results of the packaging assays performed by infection of HeLa and HuH-7 cells. The packaging efficiency is judged by the number of viral particles per ml of culture supernatant removed from the transfected PA317 packaging cells. Only those viral particles containing the vector sequences, and therefore the *neo* gene, give rise to G418-resistant HeLa or HuH-7 cells upon infection.

The LTR-modified vectors, pLCTSN and pLGCTSN, were packaged 3-10 times more efficiently than the parental pLNL6 or pLSN vectors (as judged by a comparison of the resulting titers; the titer is expressed as colony forming units (*i.e.*, G418-resistant colonies) per milliliter of harvested supernatant). These results show that the modifications made to the M-MuLV LTR do not interfere with viral replication including RNA packaging, reverse transcription and integration. In fact, the modifications to the LTR results in the modified vectors being packaged more efficiently than the parental vectors which contain unmodified M-MuLV LTRs. Similar results were obtained using stably transformed PA317 cell lines established by selection in G418 rather than transiently transfected PA317 cells (Table 3). For the experiment depicted in Table 3, HeLa cells were transduced with supernatants obtained from PA317 cells stably transformed with the indicated vectors.

TABLE 2

Titration Of Retrovirus By G418 Selection With Transfected PA317 (HuH-7, 48 hr)

Vector	pLNL6	pLSN	pLCTSN	pLGCTSN
	Titer X 10^6 [a]			
HeLa	6.5, 0.3	3.8, 9.0	22.8, 15.3	35.5, 21.0
HuH-7	2.5, 0.9, 1.0, 3.6	5.3, 0.8, 7.3, 9.7	9.0, 5.8, 7.4, 9.7	12.2, 13.8, 12.7, 12.5

[a] The titer is expressed as G418-resistant colony forming units/ml times 10^6. Thus, a value of 6.5 indicates that 6.5 x 10^6 G418-resistant colonies are produced per milliliter of harvested supernatant.

TABLE 3

Titration Of Retrovirus By G418 Selection With Infected PA317 (Producer)

Vector	pLNL6	pLSN	pLCTSN	pLGCTSN
	Titer X 10^4 [a]			
	51.6, 134	1.2, 81.4	3.6, 5.0	88.8, 6.8

[a] The titer is expressed as G418-resistant colony forming units/ml times 10^4. Thus, a value of 51.6 indicates that 51.6 x 10^4 G418-resistant colonies are produced per milliliter of harvested supernatant.

EXAMPLE 8

The pLCTSN Vector Directs Stable Expression Of Inserted Genes

In order to assess the ability of the modified LTR to direct long-term expression of genes, the long term stability of the modified vector pLCTSN was studied in HeLa and HepG2 cells. HeLa and HepG2 cells were infected (*i.e.*, transduced) with virus harvested from PA317 cells and selected with G418 as described in Example 7. Usually, cell colonies

were pooled or single cell clones picked after two weeks of growth in selective medium. These pools or single clones were then grown in selective medium for a further 1-2 months.

After 1-2 months of growth, roughly equivalent numbers of G418-resistant cells (approximately $5x10^6$ cells; typically originating from a series of different clones) were lysed and the relative CAT activity per unit protein was determined for each lysate as described in Example 1.

As shown in Figures 17 and 18, in both HeLa (Figure 17) and HepG2 cells (Figure 18), the pLCTSN-cat vector exhibited higher levels of CAT activity than did pLSN-cat, with a more pronounced difference seen in the HepG2 cells. The trans-activation effect of Tat was also assessed in the stably transduced HepG2 cells by transfecting the cells with a Tat plasmid (pCEP-tat). In this case a clear, but relatively modest level of trans-activation was observed (Figure 18). Control transfections using a β-galactosidase reporter construct suggested that less than 10% of the transduced HepG2 cells would be transfected with the Tat plasmid. Therefore, trans-activation would be expected to be at least 10-fold higher if all HepG2 cells had been transfected with the Tat plasmid. Note that the pLNL6 vector does not contain the CAT gene.

Similar results were obtained using the pLGCTSN vector in long term expression studies. The pLGCTSN vector was transduced into HeLa cells as described above for the pLCTSN vector. The transduced cells were grown in the presence of medium containing G418 for two months. G418-resistant HeLa cells containing the pLGCTSN vector were fused with a stable HeLa cell line which constitutively express the Tat protein [HeLa-*tat*-III (available from the NIH AIDS Research and Reference Reagent Program, Bethseda, MD: catalog number 502)] using polyethylene glycol 1500 (BM). This method of introducing the Tat protein is much more efficient than the transfection method employed above and results in the introduction of Tat into essentially all of the stably transduced HeLa cells. CAT assays were performed on extracts of cell lysates from the fused cells. The results of the CAT assay showed that the pLGCTSN vector stably expressed the inserted CAT gene and that expression from pLGCTSN was inducible by Tat (15 to 20-fold) in long term cultures.

These results demonstrate that the pLCTSN and pLGCTSN vectors are stable over a relatively long period (2 to 3 months) and continue to exhibit higher levels of promoter activity than the wild type M-MuLV construct.

EXAMPLE 9

Incorporation Of HIV-1 Packaging Sequences Into M-MuLV Vectors

Traditional gene therapy vectors can only infect a target cell once because the lack of M-MuLV structural proteins in the target cells precludes the packaging of vector RNA into viral particles. In some instances it would be advantageous to allow the spread of the vector genome into other cells. For example, when the vector carries genes designed to inactivate a pathogenic virus (*e.g.*, HIV), allowing the spread of the anti-viral vector sequences would increase the therapeutic value of the vector.

To further modify the improved gene therapy vectors for anti-HIV purposes, sequences in the HIV genome that are essential to genome packaging were cloned into the pLSN and pLLLgpt vectors. Upon transduction, expression of HIV packaging proteins in the target cells (*i.e.*, HIV-infected cells) will allow the assembly of the therapeutic M-MuLV vector into HIV particles thus the anti-HIV genes contained on the vector will gain access to the target HIV genome. This strategy would also help to overcome the physiological barrier of finding target RNA in a cell [Sullenger, B.A. and Cech, T.R. (1993) Science 262:1566]. This is particularly important when the anti-HIV genes contained on the therapeutic vector are ribozymes.

To permit the M-MuLV-based vectors to be packaged into HIV particles, packaging sequences derived from HIV-1 which span the psi site (near the *gag* AUG) were cloned into the pLLL and pLLLgpt vectors at the polylinker region. Two HIV-derived packaging sequences were generated. PAK100 contains approximately 100 nucleotides derived from HIV-1 and restriction recognition site for *Bam*HI at the 3' end and a cohesive overhang at the 5' end for *Sal*I. PAK100 contains the following sequence: 5'-TCGACGGATCCGCAGGAT CGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGCGGCGACTGGCATGCACGCCA AAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAAAGCTTGGATCC-3' (SEQ ID NO:10). PAK140 contains approximately 140 nucleotides derived from HIV-1 and contains the following sequence: 5'-TCGACGGATCCGCAGGATCGGCTTGCTGAAGCGCGCACG GCAAGAGGCGAGGGCGGCGACTGGCATGCACGCCAAAAATTTTGACTAGCGGAGGC TAGAAGGAGAGAAAGCTTGGATCCTAGACCGGTGCGAGAGCGTCGGTATTAAGCGG GGGAGAATTACCTAGGTGTCGACTCGCGATCGAT-3' (SEQ ID NO:11).

The PAK100 sequence was generated by annealing together the following four oligonucleotides: 5'-TCGACGGATCCGCAGGACTCGGCTTGCTGAAGCGCGCACGGCAA GAGGCGAGGGCGGCGACTGGCATG-3' (SEQ ID NO:20); 5'-CCAGTCGCCGCCCTCGC

CTCTTGCCGTGCGCGCTTCAGCAAGCCGAGTCCTGCGGATCCG-3' (SEQ ID NO:21): 5'-CACGCCAAAAATTTTGACTAGCGGAGGCTAGAAG GAGAGAAAGCTTG-3' (SEQ ID NO:22): 5'-GATCCAAGCTTTCTCTCCTTCTAGCCTCCGCTAGTCAAAATTTTTGGCG TGCATG-3' (SEQ ID NO:23). Annealing was performed as described in Example 3. This generated a 119 bp DNA fragment containing the HIV packaging signal and created 5' *Sal*I and 3' *Bam*HI restriction sites for ease of cloning. The PAK100 sequence was cloned into pLLL digested with *Xho*I-*Bam*HI digested pLLL to generate pLLL-PAK100. The PAK100 sequence was then removed from pLLL-PAK 100 by *Bam*HI digestion and inserted into the *Bam*HI site of pLLLgpt to generate pLLLgptPAK100. pLLL-PAK100 and pLLgpt-PAK100 are shown schematically in Figures 19 and 20, respectively.

The PAK140 sequence (SEQ ID NO:11) was constructed by annealing together the following two oligonucleotides: 5'-CTAGACCGGTGCGAGAGCGTCGGTATTAAGCGGGG GAGAATTACCTAGGTG-3' (SEQ ID NO:24) and 5'-TCGACACCTAGGTAATTCTCCCCC GCTTAATACCGACGCTCTCGCACCGG-3' (SEQ ID NO:25). Annealing was performed as described in Example 3. The resulting double stranded DNA fragment contains overhanging ends compatible with *Avr*II and *Sal*I; this fragment was inserted into pLLLgpt-PAK100 digested with *Avr*II and *Sal*I to generate pLLLgpt-PAK 140. pLLLgpt-PAK140 is shown schematically in Figure 21.

The ability of the viral RNA containing the synthetic HIV psi sequences to be packaged into an HIV particle inside an HIV-infected cell was tested using the following co-transfection assay.

HeLa cells were transfected with the following three plasmids: pLLLgpt-PAK140. pHIVhyg (described below) and SV-ψ'-E-MLV [Landau, N.R. and Littman, D.R. (1992) J. Virol. 66:5110]. As a control, a duplicate culture of HeLa cells was transfected with pHIVhyg.

pLLLgpt-PAK140 contains the *gpt* gene which allows for selection of cells by growth in the presence of mycophenolic acid. pLLLgpt-PAK140 contains the wild type M-MuLV LTR and HIV packaging sequences (PAK 140).

pHIVhyg contains the *hyg* gene which allows for the selection of cells by growth in the presence of hygromycin. pHIVhyg contains the HIV LTR and HIV packaging sequences. pHIVhyg expresses the HIV *gag* and *pol* genes. The *gag* gene encodes the structural components of the viral particle; the *pol* gene encodes a protease, reverse transcriptase and integrase. The expression of the HIV *gag* and *pol* gene products in the transfected HeLa cell

allows for the reverse transcription and integration of viral RNA containing the HIV LTR and the HIV packaging signal (*i.e.*, pHIVhyg).

SV-ψ⁻-E-MLV is an ecotropic M-MuLV expression vector. SV-ψ⁻-E-MLV expresses the M-MuLV *gag* and *pol* gene products. The presence of the M-MuLV *pol* gene products in the transfected HeLa cell allows the reverse transcription and integration of the pLLLgpt-PAK140 genome.

Because the transfected HeLa cells express the *gag* and *pol* gene products from both HIV and M-MuLV, the ability of pLLLgpt-PAK140 viral RNA to be packaged inside HIV particles was examined. The rational for the assay used is as follows. If pLLLgpt-PAK140 RNA is packaged into HIV particles, then the mixed particle can infect CD4⁻ cells (*i.e.*, the cell type infected by HIV). The packaging of pLLLgpt-PAK140 RNA into HIV particles would be expected to reduce the number of hyg-resistant colonies seen when HeLa CD4 are transduced with virus produced by HeLa cells co-transfected with pLLLgpt-PAK, pHIVhyg and SV-ψ⁻-E-MLV.

pHIVhyg was constructed as follows. The hygromycin B phosphotransferase (*hyg*) gene was amplified using the PCR. The PCR was performed as described in Example 2 with the exception that the following primer pair was used: 5'-GAGATATGAAAAAGCCTGAACT CAC-3' (SEQ ID NO:26) and 5'-CGCGACCGGCTGCAGAACAGCGGGC-3' (SEQ ID NO:27). The pCEP4 plasmid (Invitrogen) was used as a template for the isolation of the *hyg* sequences. The amplified *hyg* gene was inserted into pLLLSV40 digested with *Mlu*I and *Cla*I to generate pLLLSVhyg (shown schematically in Figure 22).

The SV-hyg gene fragment (*Hind*III to *Sal*I of pLLLSVhyg) was inserted between the AUG of the *nef* gene (a *Hind*III site was generated at the AUG - nucleotide 8787 of HIV_{NL43} by site-specific mutagenesis) and the *Xho*I site (nucleotide 8887) of HIV_{NL43} [Adachi, A. *et al.* (1986) J. Virol. 59:284] to generate pHIVhyg. HIV_{NL43} is a plasmid containing a wild type HIV genome. The *nef* gene was used as the site of insertion for the selectable marker because the *nef* gene is not needed for the replication of the HIV in tissue culture cells. The *nef* gene contains a unique *Xho*I site located just downstream of the ATG for the *nef* gene product. In order to avoid having the *nef* ATG upstream of the ATG for the *hyg* gene, the ATG of the *nef* gene was eliminated using site-directed mutagenesis to replace the ATG with a *Hind*III site.

The three plasmids were grown and purified as described in Example 1. HeLa cell were transfected with an equimolar ratio of each of the plasmids using the calcium phosphate co-precipitation protocol described in Example 1.

To determine the efficiency of packaging, virus was harvested from the transfected HeLa cells and was used to infect HeLa CD4 cells [Chesebro, B. *et al.* (1990) J. Virol. 64:215; HeLa CD4 cells are available from the NIH AIDS Research and Reference Reagent Program, Bethseda, MD; catalog numbers 1109 and 459]. The presence of the CD4 molecule on the surface of the cell allows for infection of the cell by virus particles containing HIV gag and env proteins. Infection was carried out as described in Example 7.

To determine whether the addition of the PAK sequences on the M-MuLV-based vector DNA interfered with the packaging of HIVhyg RNA into HIV particles, an aliquot of HeLa cells were co-transfected with pHIVhyg, pLLLgpt-PAK140 and SV-ψ⁻-E-MLV. A parallel culture of HeLa cells was co-transfected with pHIVhyg alone. Virus was harvested from the two HeLa cultures 48 hr after co-transfection. The harvested virus (1 ml of culture supernatant) was used to infect duplicate cultures of HeLa CD4 cells. Infection was carried out as described in Example 7.

The efficiency of packaging of the pHIVhyg RNA was determined by culturing the transduced HeLa CD4 cells in hygromycin-containing medium [DMEM, 10% FBS and 100 μg/ml hygromycin (Calbiochem, San Diego, CA)]. Hygromycin-resistant HeLa CD4 colonies were counted 10 to 12 days after infection. The results are summarized in Table 4.

TABLE 4

Presence of PAK Sequences Reduces Production Of Infectious HIV Particles

Vectors	Number of Hyg-Resistant HeLa CD4 colonies
pHIVhyg + pLLLgpt-PAK140 + SV-ψ⁻-E-MLV	14
pHIVhyg	39

The results shown in Table 4 show that the PAK sequences present in pLLLgpt-PAK140 interfere with the packaging of the pHIVhyg genomic RNA into HIV particles. This interference results in an almost 3-fold drop in the amount of infectious HIV particles produced by the transfected HeLa cells harboring both the HIV vector (pHIVhyg) and the

PAK vector (pLLLgpt-PAK140). These results show that the inclusion of HIV packaging sequences (*e.g.*, the PAK140 sequences) on the vector allows the vector to not only deliver genes to an HIV infected cell but also allows the vector to interfere with the packaging of the HIV genome into viral particles. Thus, the inclusion of the PAK sequences on a retroviral vector increases the therapeutic value of the vector when the vector is to be used to deliver anti-HIV genes to HIV infected cells.

In order to determine whether any of the HIV particles contain the pLLLgpt-PAK140 RNA, HeLa cells are co-transfected with equimolar amounts of pLLLgpt-PAK140, pHIVhyg and SV-ψ⁻-E-MLV. Virus is harvested and used to transduce triplicate cultures of HeLa CD4 cells. The transduced HeLa cells are grown in either XMHAT medium [DMEM, 10% FBS, 1X HAT Supplement, 250 μg/ml xanthine and 25 μg/ml mycophenolic acid; all reagents were obtained from BRL); hygromycin-containing medium (described above) or medium containing both mycophenolic acid and hygromycin. The ratio of gpt^+ to hyg^+ to gpt and hyg^+ colonies is determined. If the PAK sequences present upon pLLLgpt-PAK140 allow the vector RNA to be packaged into HIV particles and reverse transcribed then one would expect to see the same number of gpt^+ colonies (*i.e.*, capable of growth in mycophenolic acid) and gpt^+ plus hyg^+ colonies (*i.e.*, colonies capable of growth in both mycophenolic acid and hygromycin).

pLLLgpt-PAK140 was constructed to test the efficiency of packaging of M-MuLV-based vectors containing a HIV-derived packaging signal into HIV particles. For the purpose of constructing an anti-HIV gene therapy vector, the HIV psi site (either PAK100 or PAK140) is inserted into pLCTSN, which contains the improved LTR, to create pLCTSN-PAK (shown schematically in Figure 23). pLCTSN-PAK is constructed by insertion of the annealed HIV psi sequences (described above) into pLCTSN (Example 6c) digested with *Bam*HI. This design will enhance the therapeutic efficacy of the improved vectors carrying anti-HIV genes (such as anti-HIV ribozymes).

EXAMPLE 10

Selection Of High Titer Packaging Cell Clones

In order to increase the titer of recombinant virus produced by the packaging cell lines, the established PA317 and GP-AM12 [Markowitz, D. *et al.* (1988) Virol. 167:400] cell lines were subcloned to isolate those subclones which produced the highest levels of reverse

transcriptase within the starting population of packaging cells. The level of reverse transcriptase produced by the cell is an indication of the efficiency of production of the structural genes by the cell line which are needed to package the transfected recombinant vectors.

PA317 and GP-AM12 cells were plated at low density in T75 flasks (Falcon). Following three to four weeks of culturing, individual cell colonies were picked up using a cotton swab dipped in trypsin (Gibco-BRL). Ten subclones were picked from the PA317 cell line and 6 subclones were picked from the GP-AM12 cell line. The single cell clones were then grown in T25 flasks (Falcon) to confluency. An identical number of cells from each clone were cultured in a T25 flask and the supernatant was harvested at 24 hr and 48 hr. Reverse transcriptase levels were measured as follows.

A 10 µl sample of culture medium (supernatant) was incubated with 50 µl of a reaction cocktail containing 50 mM Tris-HCl, pH 8.3, 20 mM DTT, 0.6 mM $MnCl_2$, 60 mM NaCl, 0.05% NP40, 5 µg/ml of oligodeoxythymidilic acid, 10 µg/ml of polyriboadenylic acid and 10 µM of [α-^{32}P]dTTP (DuPont NEN, specific activity 800 Ci/mmol). The reaction was incubated at 37°C for 1 hr. A 3 µl aliquot was then spotted onto DE-81 paper (Whatman), and air dried. The DE-81 paper was washed 3 times in 2X SSC (20X SSC comprises: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) and autoradiographed. These results are shown in Figure 24. The clones expressing the highest levels of RT clones were chosen for use in retroviral packaging studies.

EXAMPLE 11

Retroviral Gene Therapy Vectors Containing PAK Sequences Protect Cells From HIV Infection

In order to examine the ability of the improved retroviral vectors containing PAK sequences to protect cells from infection with HIV, the following experiments were conducted. This example involved: a) transduction of H9 and AA2 cells with gene therapy vectors, b) challenge of the gene therapy-vector transduced cell lines with HIV-1 and HIV-2 and c) reverse transcriptase (RT) assay using culture supernatant from the HIV infected, transduced cells for kinetic analysis of HIV replication.

a) **Transduction Of H9 And AA2 Cells With Gene Therapy Vectors**

In order to examine the therapeutic abilities of retroviral vectors containing PAK sequences. cell lines permissive for HIV infection were first transduced with the retrovial vectors (*i.e.*., gene therapy vectors). Transduced cell lines stably expressing the genes encoded by the gene therapy vectors were selected and then used in subsequent infection studies.

Two line of cells were used for this study. H9 and AA2 cells. AA2 cells are described as an AK-. dCK- subclone of AA cells. which were derived from the WIL - 2 human splenic EBV + B-lymphoblastoid line [Chaffee. *et al. J. Exp. Med.* 168:605 (1988)]. AA-2 cells express high levels of CD4 and are remarkably permissive for HIV-1 infection and are extremely sensitive to virus cytopathic effects. AA-2 cells were grown in RPMI 1640 supplemented with non-essential amino acids and 1 mM pyruvate. and 10% fetal calf serum (all purchased from Gibco/BRL); this media is hereafter referred to as AA-2 RPMI.

To produce derivatives of AA2 that expressed the gene therapy vectors pLSNnef. pLSN-PAK140. pLCTSN-PAK140 or pLGCTSN-PAK140. AA2 cells were transduced with virus vector produced from PA317 cells.

pLSNnef contains the *nef* gene inserted into pLSN. pLSNnef was generated to serve as a negative control in these protection from infection studies. To construct pLSNnef. the *nef* gene was amplified using the PCR and the following primer pair: 5'-TTTTGCGGCCGCTCC ACCATGGGTGGCAAGTGGTC-3' (SEQ ID NO:28) and 5'-TTTTGCGGCCGCTCTAGATC AGCAGTTCTTGAAGTACTC-3' (SEQ ID NO:29). The resulting amplification product contained *Not*I sites at each end. The PCR products were digested with *Not*I and inserted into *Not*I-digested pLSN to generate pLSNnef.

pLLL-PAK100 was constructed as described in Example 9. The PAK140 sequence was inserted into the pLLLgpt-PAK100 vector (Example 9) in order to flank the PAK140 sequences with *Xho*I and *Sal*I sites; SEQ ID NOS:24 and 25 were annealed to form PAK140 (as described in Example 9) and then inserted into *Avr*II-digested pLLLgpt-PAK100 to gnerate pLLLgpt-PAK140.

pLSN-PAK140 and pLCTSN-PAK140 were derived from pLSN and pLCTSN. respectively by cloning the *Xho*I/*Sal*I fragment containing the PAK140 sequences from pLLLgpt-PAK140 into the *Xho*I site of pLSN and pLCTSN. respectively. The orientation of the PAK140 sequences in these vectors was confirmed by DNA sequencing.

pLGCTSN-PAK140 was constructed by replacing the BamHI/SalI fragment of pLGCTSN with the BglII/SalI fragment from pLCTSN-APK140 which contained the PAK140 and SV-neo sequences. The above-described plasmids are shown schematically in Figure 25.

The above-described vectors were used to transfect PA317 cells as described in Example 7 to generate PA317 cell lines producing the desired gene therapy vector (as viral particles shed into the culture media). Supernatants collected from the PA317 cells producing each vector were harvested as described in Example 7. The harvested supernatants were used to transduce AA2 cells.

In each case, 2 x 10^6 AA2 cells in T25 tissue culture flasks were exposed to 1 ml of virus supernatant harvested from PA317 virus producer cell lines and 1 ml of AA2-RPMI and 4 μg/ml of polybrene. A control or mock transduction of AA2 cells was carried in parallel using AA2-RPMI media containing 4 μg/ml of polybrene (i.e., no virus-containing supernatant was present). The cells were incubated in the presence of the virus-containing supernatants (or the control media) for 3 hr at 37°C in an incubator containing 5% CO_2. After 3 hr, each culture was supplemented with a further 3 ml of AA2-RPMI media. After a 24 hr exposure to virus, 3.5 ml of media were withdrawn from each culture flask and 3.5 ml of AA2-RPMI containing 1 mg/ml of geneticin (G418, 0-2- Amino-2-7-dideoxy-D-glycero-a-D-glucoheptopyranosyl[1->4]-0-3-deoxy-4C-methyl-3-[methyl-amino]-b-L-arabinopyronosyl-D-streptamine, purchased from Boehringer Mannheim) was added to each culture flask. G418 is used for the selection and the maintenance of eukaryotic cells stably transfected with the neomycin resistance gene (*neo* gene).

As all the gene therapy vectors used in this example contain the *neo* gene, selection of AA-2 cells after transduction with G418 produced cell lines that were also positive for expression of the gene therapy vectors. Cells were held under selection with G418 for a period of one month until it was determined that all cells in the control (*i.e.*, untransduced population) were dead. During this period of selection, the media was changed at approximately three day intervals and the G418 concentration was maintained at 1 mg/ml.

The second line of cells used for this study were H9 cells. H9 is a cloned cell line derived from a specific HUT 78 cell line. HT [Popovic, *et al.*, *Science* 224:497 (1984)]. H9 was selected for high yield permissive growth with HIV-1. The H9 cells were maintained in RPMI 1640 (90%) containing fetal calf serum (10%). H9 cells containing the three gene therapy vectors described above were transduced and selected as described for AA-2 cells above, with the following modification. H9 cells responded to the selection more rapidly than

AA-2 and as a result were only held under G418 selection for 3 weeks until it was determined that the control or mock transduced culture was dead and therefore that selection was complete.

Both H9 and AA2 cells were obtained from NIH AIDS Research and Reference Reagent Program of National Institutes of Health. Bethesda. Maryland. USA.

b) Challenge Of Gene Therapy Vector-Transduced Cell Lines With HIV-1 And HIV-2

In order to examine the ability of the gene therapy vectors to prevent or inhibit the replication of HIV. the AA-2 and H9 derived cell lines (stably transduced with the above-described gene therapy vectors) were infected with strains of HIV-1 and HIV-2 virus. The HIV-1 strains used in this example were HIV-1_{NL4-3} (NL4-3) and HIV-1_{ELI} (ELI) (HIV-1_{NL4-3} and HIV-1_{ELI} are available as catalog no. 78 and 2521. respectively from the NIH AIDS Research and Reference Reagent Program of National Institutes of Health. Bethesda. MD). The HIV-2 strain used was ROD. HIV-1_{ELI} and HIV-2 ROD were obtained from Keith Peden (U.S. Food and Drug Administration) as plasmids containing the proviral form of each virus strain (pHIVELI-1 and pROD10. respectively).

The transduced AA-2 and H9 cells were infected with the HIV strains as follows. On day zero. 2 x 10^6 cells (AA2 or H9 lines containing the desired gene therapy vector) were infected with HIV-containing cell supernatant. HIV-containing supernatant was produced as follows. HeLa cells were transfected with 20 μg of the desired plasmid DNA. Forty-eight hours later. culture supernatants were harvested and *in vitro* RT reactions were performed as described in Example 10. The titer of the virus was determined using a correlation between RT activity and MOI. A standard curve was generated using a virus stock of known titer: the resulting level of RT activity using a poly rA template (in cpm of ^{32}P-dTTP incorporated) was plotted against the known titer values. (as described below). RT activity sufficient to convert 10 cpm into the template found to be equivalent to a MOI of 1.

The HIV-infected cells (AA-2 or H9 lines) were counted and transferred to 15 ml conical tubes. The cells were then pelleted by centrifugation at 800 x *g* for 5 min. after which. they were resuspended in 1 ml of the appropriate growth media (*i.e.*. AA2-RPMI or RPMI containing 10% FCS) and viral supernatant was added to the desired multiplicity of infection (MOI). MOI by definition is the number of viral particles per cell present in an

infection. One MOI is equivalent to 10 cpm of reverse transcriptase activity [Chang, *et al.*, *J. Virol.* 67:743 (1993) and *Virology* 211:157 (1995)].

The 15 ml conical tubes containing the cells and HIV supernatants were left on their side with the caps loosened in an incubator containing 5% CO_2 for 3 hr at 37°C in order to allow the virus to infect these cells. The cells were then collected by centrifugation as described above and then resuspended in 5 ml of fresh media (either AA2-RMPI for AA2 cells or RPMI containing 10% FCS for H9 cells). The cells where then transferred to a T25 tissue culture flask and maintained in a 37°C, 5% CO_2 incubator for the duration of the study.

c) RT Assay Using Culture Supernatant From The HIV Infected, Transduced Cells For Kinetic Analysis Of HIV Replication

At alternating intervals of 4 and 3 days, 3.5 ml of culture media were withdrawn from the T25 flasks containing the HIV-infected cells which were transduced with the desired gene therapy vector. Approximately 200 µl was removed from the 3.5 ml and was stored by freezing at -80°C for reverse transcriptase assay and the remainder was discarded. The media volume that had been withdrawn was replaced by the addition of fresh media. At the end of the study period (29-39 days), the harvested cell supernatants collected at 3-4 day intervals were thawed and the reverse transcriptase assay was performed as described in Example 10. The level of RT activity present is a measure of the amount of infectious HIV particles shed into the culture supernatant from each infected culture. The results of these experiments are summarized in Figures 26 and 27.

In Figure 26, Panel A shows the cpm/µl of culture supernatant harvested from H9 cells containing various gene therapy vectors plotted against the number of days post-infection with HIV_{NL4-3}. The HIV infection was carried out using a MOI of 0.001. The open circles represent the results obtained using culture supernatant harvested from uninfected H9 cells (*i.e.*, the negative control for the RT assay). The closed circles represent the results obtained using culture supernatant harvested from H9 cells (not transduced with a gene therapy vector) infected with HIV-1 (*i.e.*, the positive control). The open triangles represent the results obtained using culture supernatant harvested from H9 cells transduced with pLSNnef vector and infected with HIV-1. The closed triangles represent the results obtained using culture supernatant harvested from H9 cells transduced with pLSN-PAK140 vector and infected with HIV-1. The open squares represent the results obtained using culture supernatant harvested from H9 cells transduced with pLCTSN-PAK140 vector and infected with HIV-1. The closed

squares represent the results obtained using culture supernatant harvested from H9 cells transduced with pLGCTSN-PAK140 vector and infected with HIV-1.

In Figure 26, Panel B shows the cpm/μl of culture supernatant harvested from AA2 cells containing various gene therapy vectors plotted against the number of days post-infection with HIV_{NL4-3}. The HIV infection was carried out using a MOI of 0.001. The open circles represent the results obtained using culture supernatant harvested from uninfected AA2 cells (*i.e.*, the negative control for the RT assay). The closed circles represent the results obtained using culture supernatant harvested from AA2 cells (not transduced with a gene therapy vector) infected with HIV-1 (*i.e.*, the positive control). The open triangles represent the results obtained using culture supernatant harvested from AA2 cells transduced with pLSNnef vector and infected with HIV-1. The closed triangles represent the results obtained using culture supernatant harvested from AA2 cells transduced with pLSN-PAK140 vector and infected with HIV-1. The open squares represent the results obtained using culture supernatant harvested from AA2 cells transduced with pLCTSN-PAK140 vector and infected with HIV-1. The closed squares represent the results obtained using culture supernatant harvested from AA2 cells transduced with pLGCTSN-PAK140 vector and infected with HIV-1.

In Figure 27, Panel A shows the cpm/μl of culture supernatant harvested from H9 cells containing various gene therapy vectors plotted against the number of days post-infection with HIV_{ELI}. The HIV infection was carried out using a MOI of 0.2. The open circles represent the results obtained using culture supernatant harvested from uninfected H9 cells (*i.e.*, the negative control for the RT assay). The closed circles represent the results obtained using culture supernatant harvested from H9 cells (not transduced with a gene therapy vector) infected with HIV-1-ELI (*i.e.*, the positive control). The open triangles represent the results obtained using culture supernatant harvested from H9 cells transduced with pLSNnef vector and infected with HIV-1-ELI. The closed triangles represent the results obtained using culture supernatant harvested from H9 cells transduced with pLSN-PAK140 vector and infected with HIV-1-ELI. The open squares represent the results obtained using culture supernatant harvested from H9 cells transduced with pLCTSN-PAK140 vector and infected with HIV-1-ELI. The closed squares represent the results obtained using culture supernatant harvested from H9 cells transduced with pLGCTSN-PAK140 vector and infected with HIV-1-ELI.

In Figure 27, Panel B shows the cpm/μl of culture supernatant harvested from H9 cells containing various gene therapy vectors plotted against the number of days post-infection with HIV_{ROD}. The HIV infection was carried out using a MOI of 0.2. The open circles represent the results obtained using culture supernatant harvested from uninfected H9 cells (*i.e.*, the negative control for the RT assay). The closed circles represent the results obtained using culture supernatant harvested from H9 cells (not transduced with a gene therapy vector) infected with HIV-2-ROD (*i.e.*, the positive control). The closed triangles represent the results obtained using culture supernatant harvested from H9 cells transduced with pLSN-PAK140 vector and infected with HIV-2-ROD. The closed squares represent the results obtained using culture supernatant harvested from H9 cells transduced with pLGCTSN-PAK140 vector and infected with HIV-2-ROD.

The results shown in Figures 26 and 27 demonstrate that the presence of the pLGCTSN-PAK140 vector protects AA2 or H9 cells from infection by HIV. These studies demonstrate that the novel anti-HIV vector pLGCTSN-PAK140 can block infection by HIV-1 and HIV-2 up to 100% in different human $CD4^-$ lymphoid cell lines for longer than 40 days. In this example, the ability of cells containing the pLGCTSN-PAK140 vector to block invention by HIV was compared with cells containing a conventional MLV vector, pLSN, which either lacked (*i.e.*, pLSNnef) or contained (*i.e.*, pLSN-PAK140) the PAK140 sequences on the vector. The results obtained clearly indicated that the further modifications of the vector in the LTR and the packaging (psi) regions contained within pLGCTSN-PAK140 are critical for the anti-HIV effect displayed by pLGCTSN-PAK140 as the presence of pLSN-PAK140 in H9 or AA2 cells had no effect upon the ability of $HIV\text{-}1_{NL4\text{-}3}$, $HIV\text{-}1_{ELI}$ or HIV-2 ROD to infect these cells (*See* Figures 26 and 27). Thus, the novel Tat-inducible LTR and the extended packaging sequences (*i.e.*, extended packaging signal) and the intron contained on the pLGCTSN vector provide features necessary for the generation of a successful anti-HIV gene therapy vector.

While not limiting the invention to any particular theory, the inhibitory effects of pLGCTSN-PAK140 may occur at the transcriptional level where competition for Tat may reduce HIV expression; alternatively the inhibitory effects may occur at the level of virion assembly as the pLGCTSN-PAK140 transcript may compete for packaging into the HIV particle. In addition, the above examples demonstrate that the pLGCTSN vector expresses the encoded genes at higher levels in comparison to the use of conventional MLV vectors (*e.g.*,

pLSN). In addition, expression of genes from the pLGCTSN vector is more stable in comparison to expression of genes contained within conventional MLV vectors (*e.g.*, pLSN).

The pLGCTSN-PAK140 RNA can be packaged into HIV particles. The resulting chimeric particles (*i.e.*, pLGCTSN-PAK140 RNA inside the HIV viral coat) released from the HIV-infected cell are non-infectious; these chimeric particles may be immunogenic (*i.e.*, capable of eliciting an immune response directed against HIV proteins). Therefore the use of the pLGCTSN-PAK140 vector would be advantageous in HIV gene therapy protocols. Peripheral blood lymphocytes or $CD34^+$ enriched lymphocytes from HIV-infected patients are isolated and transduced with the pLGCTSN-PAK140 virus; the transduced lymphocytes are then infused into the patient. The transduced cells may show resistance to infection by HIV and if already infected, the transduced cells may suppress replication of HIV. The small population of lymphocytes transduced by the gene therapy vector pLGCTSN-PAK140 may expand in the patient with time due to the rapid killing of HIV-infected cells due to the cytopathetic effects of HIV. Therefore, even if transduction of human lymphocytes by the novel anti-HIV gene therapy viruses of the present invention occurs with low efficiency, this may not present problems for HIV gene therapy. In addition, minor effects on HIV replication *in vivo* may have a great impact upon development of disease in the patient long term.

The results shown above using the novel pLGCTSN-PAK140 vector demonstrate that this vector can be used to block HIV infection of $CD4^+$ human cell lines. The results obtained in this example show that the pLGCTSN-PAK140 gene therapy vector is superior to other anti-HIV vectors reported in the literature [Aguilar-Cordova, *et al.* (1995) Gene Therapy 2:181; Escaich, *et al.*(1995) Human Gene Therapy, 6:625; Lee, *et al.* (1995) Gene Therapy (1995) 2:377 and Lori, *et al.* (1994) Gene Therapy 1:27].

The novel anti-HIV gene therapy vectors of the present invention (*e.g.*, pLGCTSN, pLGCTSN-PAK140) may be modified to permit the insertion of other anti-HIV genes into these vectors to permit even greater effects upon the ability to block HIV replication in cells containing these vectors.

From the above examples it should be clear that the improved retroviral vectors of the invention, comprising novel LTRs and extended MuMLV packaging sequences, provide for the efficient packaging of vector RNA and the efficient long-term expression of genes inserted into the improved vectors. The improved promoters found in the novel LTRs obviate the need to use an internal promoter to drive the expression of inserted genes. The inserted genes

are expressed at high levels which enables the study of gene expression. cell lineage analysis in a wide variety of cell lines. The improved promoters function in a wide variety of human cell types making vectors containing these promoters ideal for the delivery of genes to a variety of cell lines and tissues. The vectors containing the novel LTRs and HIV-1 packaging sequences provide an improved means of delivering anti-HIV genes. The presence of the novel HIV-1 packaging sequences on the vectors of the present invention provides a means to protect cells from infection by HIV.

SEQUENCE LISTING

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(i) APPLICANT: CHANG, LUNG-JI

(ii) TITLE OF INVENTION: RETROVIRAL VECTORS

(iii) NUMBER OF SEQUENCES: 29

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(C) CITY: SAN FRANCISCO
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(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 94104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/336,132
(B) FILING DATE: 08-NOV-1994

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 32,837
(C) REFERENCE/DOCKET NUMBER: CHANG-01999

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6145 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCATAC CAGATCACCG AAAACTGTCC TCCAAATGTG TCCCCCTCAC ACTCCCAAAT 60

TCGCGGGCTT CTGCCTCTTA GACCACTCTA CCCTATTCCC CACACTCACC GGAGCCAAAG 120

CCGCGGCCCT TCCGTTTCTT TGCTTTTGAA AGACCCCACC CGTAGGTGGC AAGCTAGCTT 180

AAGTAACGCC ACTTTGCAAG GCATGGAAAA ATACATAACT GAGAATAGAA AAGTTCAGAT 240

CAAGGTCAGG AACAAAGAAA CAGCTGAATA CCAAACAGGA TATCTGTGGT AAGCGGTTCC 300

TGCCCCGGCT CAGGGCCAAG AACAGATGAG ACAGCTGAGT GATGGGCCAA ACAGGATATC 360

TGTGGTAAGC AGTTCCTGCC CCGGCTCGGG GCCAAGAACA GATGGTCCCC AGATGCGGTC 420

CAGCCCTCAG CAGTTTCTAG TGAATCATCA GATGTTTCCA GGGTGCCCCA AGGACCTGAA 480

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AATGACCCTG TACCTTATTT GAACTAACCA ATCAGTTCGC TTCTCGCTTC TGTTCGCGCG     540
CTTCCGCTCT CCGAGCTCAA TAAAAGAGCC CACAACCCCT CACTCGGCGC GCCAGTCTTC     600
CGATAGACTG CGTCGCCCGG GTACCCGTAT TCCCAATAAA GCCTCTTGCT GTTTGCATCC     660
GAATCGTGGT CTCGCTGTTC CTTGGGAGGG TCTCCTCTGA GTGATTGACT ACCCACGACG     720
GGGGTCTTTC ATTTGGGGGC TCGTCCGGGA TTTGGAGACC CCTGCCCAGG GACCACCGAC     780
CCACCACCGG GAGGTAAGCT GGCCAGCAAC TTATCTGTGT CTGTCCGATT GTCTAGTGTC     840
TATGTTTGAT GTTATGCGCC TGCGTGTGTA CTAGTTAGCT AACTAGCTCT GTATCTGGCG     900
GACCCGTGGT GGAACTGACG AGTTCTGAAC ACCCGGCCGC AACCCTGGGA GACGTCCCAG     960
GGACTTTGGG GGCCGTTTTT GTGGCCCGAC CTGAGGAAGG GAGTCGATGT GGAATCCGAC    1020
CCCGTCAGGA TATGTGGTTC TGGTAGGAGA CGAGAACCTA AAACAGTTCC CGCCTCCGTC    1080
TGAATTTTTG CTTTCGGTTT GGAACCGAAG CCGCGCGTCT TGTCTGCTGC AGCGCTGCAG    1140
CATCGTTCTG TGTTGTCTCT GTCTGACTGT GTTTCTGTAT TTGTCTGAAA ATTAGGGCCA    1200
GACTGTTACC ACTCCCTTAA GTTTGACCTT AGGTCACTGG AAAGATGTCG AGCGGATCGC    1260
TCACAACCAG TCGGTAGATG TCAAGAAGAG ACGTTGGGTT ACCTTCTGCT CTGCAGAATG    1320
GCCAACCTTT AACGTCGGAT GGCCGCGAGA CGGCACCTTT AACCGAGACC TCATCACCCA    1380
GGTTAAGATC AAGGTCTTTT CACCTGGCCC GCATGGACAC CCAGACCAGG TCCCCTACAT    1440
CGTGACCTGG GAAGCCTTGG CTTTTGACCC CCCTCCCTGG GTCAAGCCCT TTGTACACCC    1500
TAAGCCTCCG CCTCCTCTTC CTCCATCCGC CCCGTCTCTC CCCCTTGAAC CTCCTCGTTC    1560
GACCCCGCCT CGATCCTCCC TTTATCCAGC CCTCACTCCT TCTCTAGGCG CCGGAATTCC    1620
GATCTGATCA AGAGACAGGA TGAGGATCGT TTCGCATGAT TGAACAAGAT GGATTGCACG    1680
CAGGTTCTCC GGCCGCTTGG GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA    1740
TCGGCTGCTC TGATGCCGCC GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG    1800
TCAAGACCGA CCTGTCCGGT GCCCTGAATG AACTGCAGGA CGAGGCAGCG CGGCTATCGT    1860
GGCTGGCCAC GACGGGCGTT CCTTGCGCAG CTGTGCTCGA CGTTGTCACT GAAGCGGGAA    1920
GGGACTGGCT GCTATTGGGC GAAGTGCCGG GGCAGGATCT CCTGTCATCT CACCTTGCTC    1980
CTGCCGAGAA AGTATCCATC ATGGCTGATG CAATGCGGCG GCTGCATACG CTTGATCCGG    2040
CTACCTGCCC ATTCGACCAC CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG    2100
AAGCCGGTCT TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG    2160
AACTGTTCGC CAGGCTCAAG GCGCGCATGC CCGACGGCGA GGATCTCGTC GTGACCCATG    2220
GCGATGCCTG CTTGCCGAAT ATCATGGTGG AAAATGGCCG CTTTTCTGGA TTCATCGACT    2280
GTGGCCGGCT GGGTGTGGCG GACCGCTATC AGGACATAGC GTTGGCTACC CGTGATATTG    2340
CTGAAGAGCT TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC    2400
CCGATTCGCA GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT    2460
GGGGTTCGAA ATGACCGACC AAGCGACGCC CAACCTGCCA TCACGAGATT TCGATTCCAC    2520
CGCCGCCTTC TATGAAAGGT TGGGCTTCGG AATCGTTTTC CGGGACGCCG GCTGGATGAT    2580
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CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCGGGCTCG ATCCCCTCGC 2640

GAGTTGGTTC AGCTGCTGCC TGAGGCTGGA CGACCTCGCG GAGTTCTACC GGCAGTGCAA 2700

ATCCGTCGGC ATCCAGGAAA CCAGCAGCGG CTATCCGCGC ATCCATGCCC CCGAACTGCA 2760

GGAGTGGGGA GGCACGATGG CCGCTTTGGT CGACCCGGAC GGGACGCTCC TGCGCCTGAT 2820

ACAGAACGAA TTGCTTGCAG GCATCTCATG AGTGTGTCTT CCCGTTTTCC GCCTGAGGTC 2880

ACTGCGTGGA TGGAGCGCTG GCGCCTGCTG CGCGACGGCG AGCTGCTCAC CACCCACTCG 2940

AGGGCGTGCA GCGCTGCAGA GGCCGAGTGC AGAACTGCTC CAAAGGGACC TCAAGGCTTT 3000

CCGAGGGACA CTAGGCTGAC TCCATCGAGC CAGTGTAGAG ATAAGCTTAT CGATTAGTCC 3060

AATTTGTTAA AGACAGGATA TCAGTGGTCC AGGCTCTAGT TTTGACTCAA CAATATCACC 3120

AGCTGAAGCC TATAGAGTAC GAGCCATAGA TAAAATAAAA GATTTTATTT AGTCTCCAGA 3180

AAAAGGGGGG AATGAAAGAC CCCACCTGTA GGTTTGGCAA GCTAGCTTAA GTAACGCCAT 3240

TTTGCAAGGC ATGGAAAAAT ACATAACTGA GAATAGAGAA GTTCAGATCA AGGTCAGGAA 3300

CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT GGTAAGCAGT TCCTGCCCCG 3360

GCTCAGGGCC AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA 3420

GCAGTTCCTG CCCCGGCTCA GGGCCAAGAA CAGATGGTCC CCAGATGCGG TCCAGCCCTC 3480

AGCAGTTTCT AGAGAACCAT CAGATGTTTC CAGGGTGCCC CAAGGACCTG AAATGACCCT 3540

GTGCCTTATT TGAACTAACC AATCAGTTCG CTTCTCGCTT CTGTTCGCGC GCTTCTGCTC 3600

CCCGAGCTCA ATAAAAGAGC CCACAACCCC TCACTCGGGG CGCCAGTCCT CCGATTGACT 3660

GAGTCGCCCG GGTACCCGTG TATCCAATAA ACCCTCTTGC AGTTGCATCC GACTTGTGGT 3720

CTCGCTGTTC CTTGGGAGGG TCTCCTCTGA GTGATTGACT ACCCGTCAGC GGGGGTCTTT 3780

CATTTGGGGG CTCGTCCGGG ATCGGGAGAC CCCTGCCCAG GGACCACCGA CCCACCACCG 3840

GGAGGTAAGC TGGCTGCCTC GCGCGTTTCG GTGATGACGG TGAAAACCTC TGACACATGC 3900

AGCTCCCGGA GACGGTCACA GCTTGTCTGT AAGCGGATGC CGGGAGCAGA CAAGCCCGTC 3960

AGGGCGCGTC AGCGGGTGTT GGCGGGTGTC GGGGCGCAGC CATGACCCAG TCACGTAGCG 4020

ATAGCGGAGT GTATACTGGC TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA 4080

CCATATGCGG TGTGAAATAC CGCACAGATG CGTAAGGAGA AAATACCGCA TCAGGCGCTC 4140

TTCCGCTTCC TCGCTCACTG ACTCGCTGCG CTCGGTCGTT CGGCTGCGGC GAGCGGTATC 4200

AGCTCACTCA AAGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG CAGGAAAGAA 4260

CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT 4320

TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG 4380

GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG 4440

CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG 4500

CGTGGCGCTT TCTCATAGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC 4560

CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA 4620

CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG 4680

TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA AGTGGTGGCC 4740

TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC 4800

CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG 4860

TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT 4920

GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT 4980

CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA 5040

ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT TAATCAGTGA 5100

GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCTGAC TCCCCGTCGT 5160

GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG 5220

AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA 5280

GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCGGGA 5340

AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTTGCCA TTGCTGCAGG 5400

CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC 5460

AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC 5520

GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA 5580

TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC 5640

CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAACACG 5700

GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC 5760

GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG 5820

TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC 5880

AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT GAATACTCAT 5940

ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA 6000

CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT TTCCCCGAAA 6060

AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG 6120

TATCACGAGG CCCTTTCGTC TTCAA 6145

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCTAAGCT TGCGGCCGCA GATCTCGAGC CATGGATCCT AGGCCTGATC ACGCGTCGAC 60

TCGCGAT 67

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGATCGCGAG TCGACGCGTG ATCAGGCCTA GGATCCATGG CTCGAGATCT GCGGCCGCAA 60

GCTTA 65

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGCTTGATC ACCACCATGA TTGAACAAGA TGG 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCGGATCCGT CGACCCCAGA GTCCCGCTCA GAAG 34

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCATCTAGAG TACTTCAAGA ACTGC 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAATTCGA GGCTTAAGCA GTGGGTTCC 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCGGAGTAGC TAGCTGGAGT TCCGC 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTAGCGGTA CC 12

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGACGGATC CGCAGGATCG GCTTGCTGAA GCGCGCACGG CAAGAGGCGA GGGCGGCGAC 60

TGGCATGCAC GCCAAAAATT TTGACTAGCG GAGGCTAGAA GGAGAGAAAG CTTGGATCC 119

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCGACGGATC CGCAGGATCG GCTTGCTGAA GCGCGCACGG CAAGAGGCGA GGGCGGCGAC 60

TGGCATGCAC GCCAAAAATT TTGACTAGCG GAGGCTAGAA GGAGAGAAAG CTTGGATCCT 120

AGACCGGTGC GAGAGCGTCG GTATTAAGCG GGGGAGAATT ACCTAGGTGT CGACTCGCGA 180

TCGAT 185

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGGATCCTC GAGCCACCAT GGAGCCAGTA GATCCT 36

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAAGATCTGC ATGCTAATCG AACGGATCTG TC 32

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCTAGAAGC TTAGTGCGCC AGATCTCTAT AATC 34

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATCTAGACTC GAGTTAGCGA CCGGAGATTG GC 32

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 702 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATGAAAGAC CCCACCTGTA GGTTTGGCAA GCTAGCTTAA GTAACGCCAT TTTGCAAGGC 60

ATGGAAAAAT ACATAACTGA GAATAGAGAA GTTCAGATCA AGGTCAGGAA CAGATGGAAC 120

AGCTGAATAT GGGCCAAACA GGATATCTGT GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC 180

AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG 240

CCCCGGCTCA GGGCCAAGAA CAGATGGTCC CCAGATGCGG TCCAGCCCTC AGCAGTTTCT 300

AGAGTACTTC AAGAACTGCT GACATCGAGC TTGCTACAAG GGACTTTCCG CTGGGGACTT 360

TCCAGGGAGG CGTGGCCTGG GCGGGACTGG GGAGTGGCGA GCCCTCAGAT GCTGCATATA 420

AGCAGCTGCT TTTTGCCTGT ACTGGGTCTC TCTGGTTAGA CCAGATCTGA GCCTGGGAGC 480

TCTCTGGCTA ACTAGGGAAC CCACTGCTTA AGCCTCGAAT TCAGCTCAAT AAAAGAGCCC 540

ACAACCCCTC ACTCGGGGCG CCAGTCCTCC GATTGACTGA GTCGCCCGGG TACCCGTGTA 600

TCCAATAAAC CCTCTTGCAG TTGCATCCGA CTTGTGGTCT CGCTGTTCCT TGGGAGGGTC 660

TCCTCTGAGT GATTGACTAC CCGTCAGCGG GGGTCTTTCA TT 702

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 825 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATGAAAGAC CCCACCTGTA GGTTTGGCAA GCTAGCTTAA GTAACGCCAT TTTGCAAGGC 60

ATGGAAAAAT ACATAACTGA GAATAGAGAA GTTCAGATCA AGGTCAGGAA CAGATGGAAC 120

AGCTGAATAT GGGCCAAACA GGATATCTGT GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC 180

AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG 240

CCCCGGCTCA GGGCCAAGAA CAGATGGTCC CCAGATGCGG TCCAGCCCTC AGCAGTTTCT 300

AGCTGGAGTT CCGCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA CCGCCCAACG 360

ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT AGTAACGCCA ATAGGGACTT 420

TCCATTGACG TCAATGGGAG TTTGTTTTGG CACCAAAATC AACGGGACTT TCCAAAATGT 480

CGTAATAACC CCGCCCCGTT GACGCAAATG GGCGGTAGGC GTGTACTCTA GATGCTACAT 540

ATAAGCAGCT GCTTTTTGCC TGTACTGGGT CTCTCTGGTT AGACCAGATC TGAGCCTGGG 600

AGCTCTCTGG CTAACTAGGG AACCCACTGC TTAAGCCTCG AATTCAGCTC AATAAAAGAG 660

CCCACAACCC CTCACTCGGG GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT 720

GTATCCAATA AACCCTCTTG CAGTTGCATC CGACTTGTGG TCTCGCTGTT CCTTGGGAGG 780

GTCTCCTCTG AGTGATTGAC TACCCGTCAG CGGGGGTCTT TCATT 825

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAACCTCCTC GTTCGACC 18

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AACTAGAGCC TGGACCAC 18

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCGACGGATC CGCAGGACTC GGCTTGCTGA AGCGCGCACG GCAAGAGGCG AGGGCGGCGA 60

CTGGCATG 68

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCAGTCGCCG CCCTCGCCTC TTGCCGTGCG CGCTTCAGCA AGCCGAGTCC TGCGGATCCG 60

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CACGCCAAAA ATTTTGACTA GCGGAGGCTA GAAGGAGAGA AAGCTTG 47

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATCCAAGCT TTCTCTCCTT CTAGCCTCCG CTAGTCAAAA TTTTTGGCGT GCATG 55

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTAGACCGGT GCGAGAGCGT CGGTATTAAG CGGGGGAGAA TTACCTAGGT G 51

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCGACACCTA GGTAATTCTC CCCCGCTTAA TACCGACGCT CTCGCACCGG 50

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAGATATGAA AAAGCCTGAA CTCAC 25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGCGACCGGC TGCAGAACAG CGGGC 25

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTTTGCGGCC GCTCCACCAT GGGTGGCAAG TGGTC 35

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTTGCGGCC GCTCTAGATC AGCAGTTCTT GAAGTACTC 39

CLAIMS

1. A recombinant Moloney murine leukemia virus long terminal repeat which is activated by the human immunodeficiency virus 1 Tat protein, said recombinant long terminal repeat having increased promoter activity relative to the wild type Moloney murine leukemia virus long terminal repeat in human cells.

2. The recombinant long terminal repeat of Claim 1, wherein said long terminal repeat contains the human cytomegalovirus immediate early enhancer/promoter and the HIV-1 TATA and TAR elements in place of the Moloney murine leukemia virus promoter element in the U3 region of said long terminal repeat.

3. The recombinant long terminal repeat of Claim 2 having the sequence shown in SEQ ID NO:17.

4. The recombinant long terminal repeat of Claim 1, wherein said long terminal repeat is contained on a vector.

5. The recombinant long terminal repeat of Claim 4, wherein said vector containing said long terminal repeat is contained within a host cell.

6. The host cell of Claim 5 wherein said host cell is a bacterial host cell.

7. The host cell of Claim 5 wherein said host cell is a human cell.

8. The recombinant long terminal repeat of Claim 4 wherein said vector is a recombinant murine amphotropic retroviral vector, said retroviral vector comprising in operable order:

a) a first long terminal repeat;

b) a packaging signal joined to said first long terminal repeat; and

c) a second long terminal repeat joined to said packaging signal.

9. The recombinant long terminal repeat of Claim 6, wherein said vector further comprises an oligonucleotide having a nucleotide sequence encoding a selectable marker gene, wherein said selectable marker gene is operably linked between said packaging signal and said second long terminal repeat.

10. The recombinant long terminal repeat of Claim 7, wherein said selectable marker gene is a dominant selectable marker gene.

11. The recombinant long terminal repeat of Claim 8, wherein said dominant selectable marker gene is the neomycin phosphoribosyltransferase gene.

12. The recombinant long terminal repeat of claim 9 wherein the vector comprises pLCTSN.

13. The recombinant long terminal repeat of Claim 6 wherein said packaging signal comprises an extended Moloney murine leukemia virus packaging signal, said extended packaging signal resulting in an increased packaging efficiency of said recombinant vector.

14. The recombinant long terminal repeat of Claim 11 wherein the vector comprises pLGCTSN.

15. The recombinant long terminal repeat of Claim 6 wherein said packaging signal comprises a human immunodeficiency virus packaging signal.

16. The recombinant retroviral vector of Claim 13 wherein said human immunodeficiency virus packaging signal consists of the sequence listed in SEQ ID NO:10.

17. The recombinant retroviral vector of Claim 13 wherein said human immunodeficiency virus packaging signal consists of the sequence listed in SEQ ID NO:11.

18. A recombinant murine amphotropic retroviral vector containing the following elements in operable order:

a) a first long terminal repeat:

b) a packaging signal joined to said first long terminal repeat:

c) a polylinker joined to said packaging signal: and

d) a second long terminal repeat consisting of the sequence listed in SEQ ID NO:17 joined to said polylinker.

19. The recombinant vector of Claim 16 further comprising a selectable marker inserted into said polylinker.

20. The recombinant vector of Claim 17 wherein said selectable marker is a dominant selectable marker.

21. The recombinant vector of Claim 18 wherein said dominant selectable marker is the neomycin phosphoribosyltransferase gene.

22. The recombinant vector of Claim 16 wherein said packaging signal comprises an extended Moloney murine leukemia virus packaging signal.

23. The recombinant vector of Claim 18 wherein said packaging signal comprises a packaging signal derived from human immunodeficiency virus 1.

24. The recombinant vector of Claim 21 wherein said packaging signal consists of SEQ ID NO:10.

25. The recombinant vector of Claim 21 wherein said packaging signal consists of SEQ ID NO:11.

26. The recombinant retroviral vector pLLL.

27. A method for expressing a gene in a human cell line, said method comprising:

a) providing:

i) a human cell line; and

ii) a retroviral vector containing the recombinant long terminal repeat of SEQ ID NO:17 and a gene of interest; and

b) introducing said retroviral vector into said human cell line under conditions which allow the expression of said gene of interest.

28. The method of Claim 32 wherein said vector further contains a selectable marker.

29. The method of Claim 36 wherein said selectable marker is a dominant selectable marker.

30. The method of Claim 37 wherein said dominant selectable marker is the neomycin phosphoribosyltransferase gene.

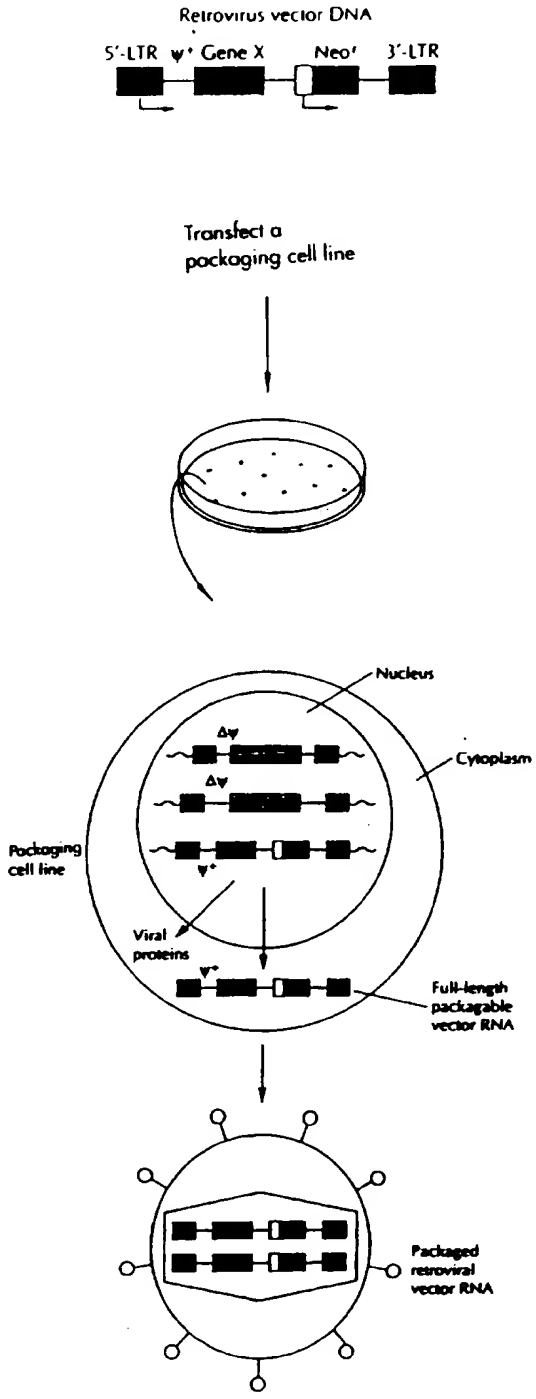
31. The method of Claim 36 which comprises the further step:

c) exposing said human cell line to conditions, wherein said conditions allow only those human cells expressing said selectable marker to grow.

32. The method of Claim 39 wherein said conditions comprise a selective medium.

33. The method of Claim 40 wherein said selective medium contains the antibiotic G418.

FIGURE 1


Retrovirus vector DNA
5'-LTR
ψ+
Gene X
Neo'
3'-LTR
Transfect a packaging cell line
Nucleus
Δψ
Δψ
Cytoplasm
Packaging cell line
ψ+
Viral proteins
ψ+
Full-length packagable vector RNA
Packaged retroviral vector RNA

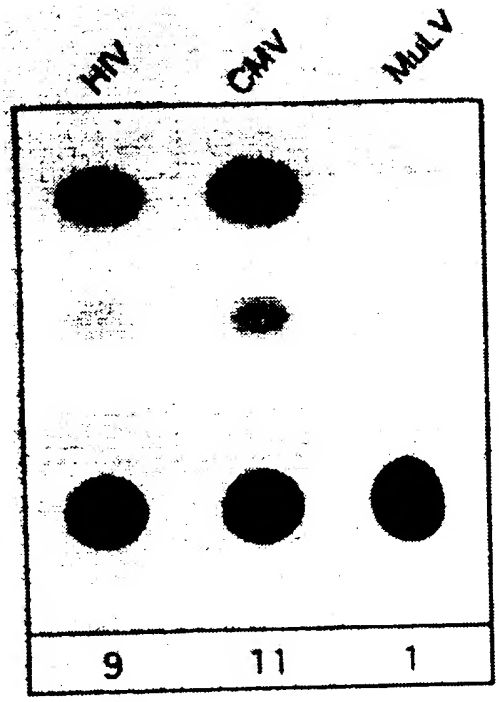


FIG. 2

FIGURE 3

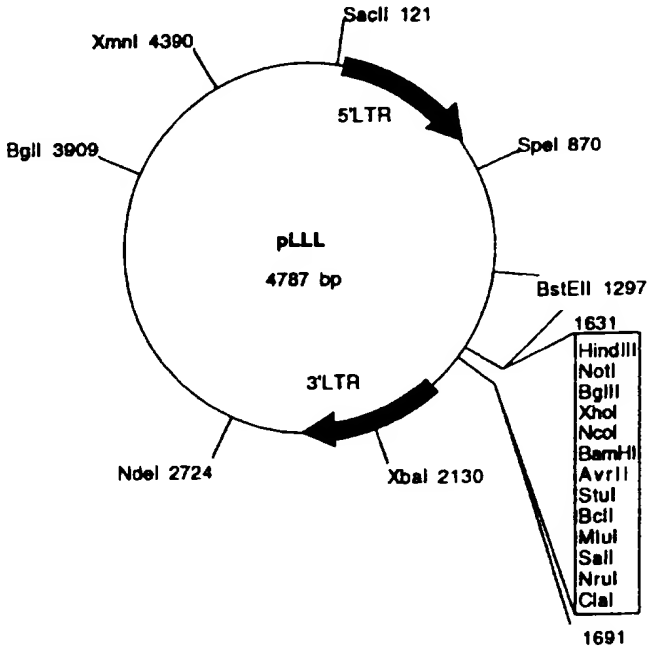

SacII 121
XmnI 4390
5'LTR
SpeI 870
BglI 3909
pLLL
4787 bp
BstEII 1297
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
AvrII
StuI
BclI
MluI
SalI
NruI
ClaI
3'LTR
NdeI 2724
XbaI 2130
1691

FIGURE 4

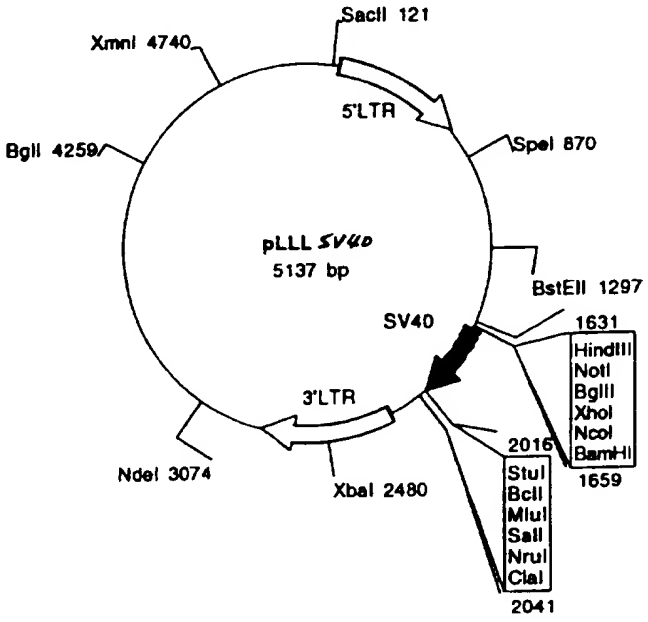

SacII 121
Xmnl 4740
5'LTR
Bgll 4259
SpeI 870
pLLL SV40
5137 bp
BstEII 1297
SV40
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
1659
3'LTR
2016
StuI
BclI
MluI
SalI
NruI
ClaI
2041
NdeI 3074
XbaI 2480

FIGURE 5

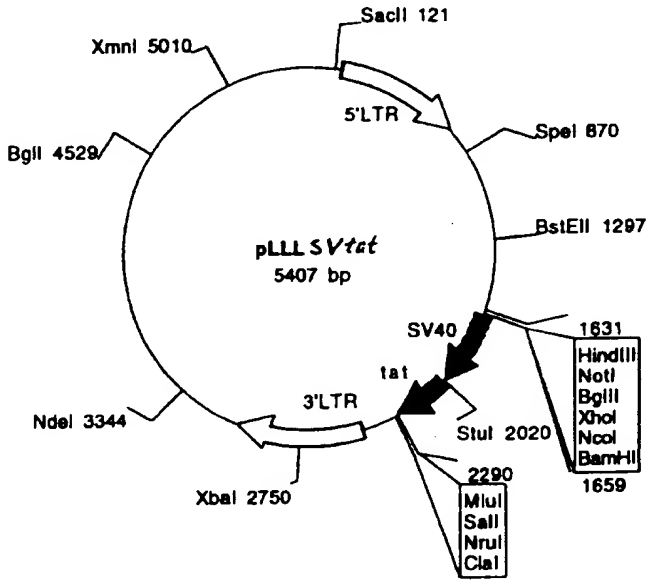

SacII 121
Xmnl 5010
5'LTR
SpeI 870
Bgll 4529
BstEII 1297
pLLLSVtat
5407 bp
SV40
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
1659
tat
3'LTR
Ndel 3344
StuI 2020
2290
MluI
SalI
NruI
ClaI
Xbal 2750

FIGURE 6

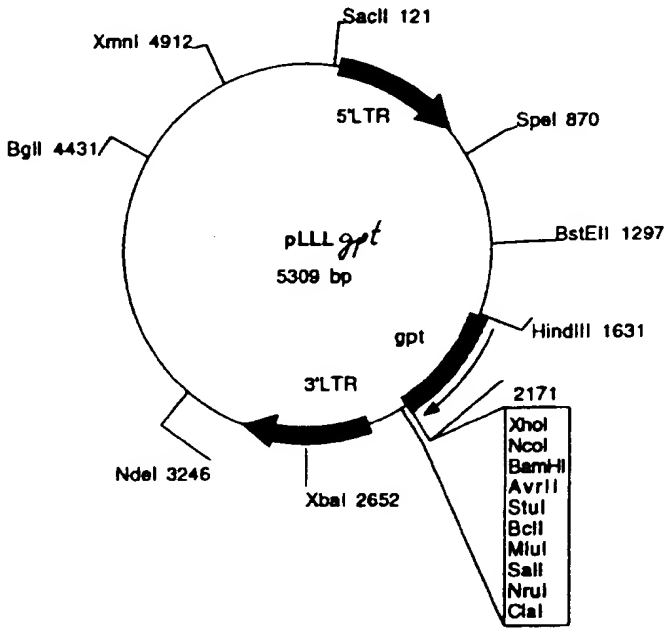

SacII 121
XmnI 4912
5'LTR
SpeI 870
BglI 4431
pLLL gpt
5309 bp
BstEII 1297
gpt
HindIII 1631
3'LTR
2171
XhoI
NcoI
BamHI
AvrII
StuI
BclI
MluI
SalI
NruI
ClaI
NdeI 3246
XbaI 2652

FIGURE 7

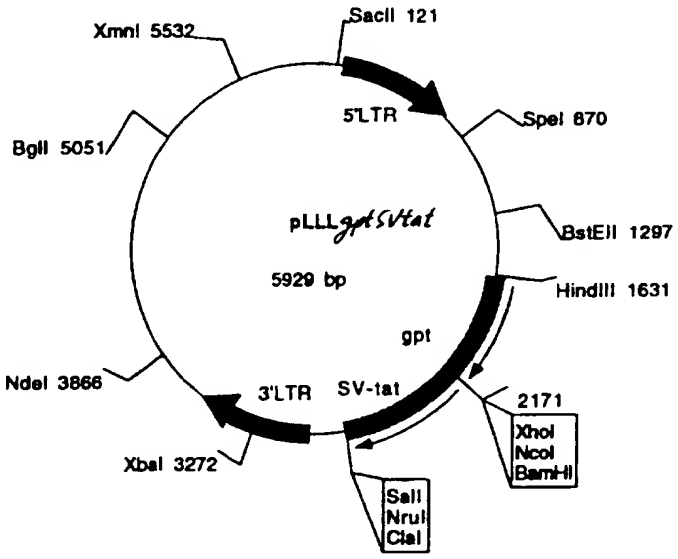
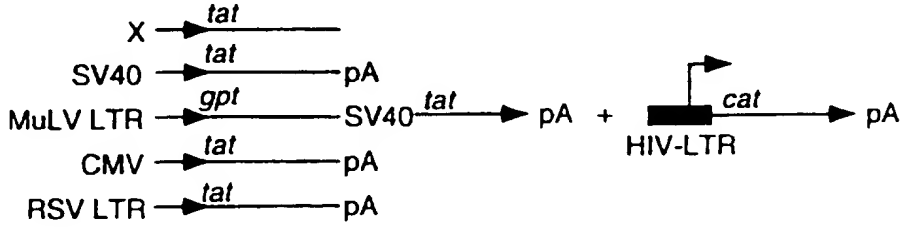
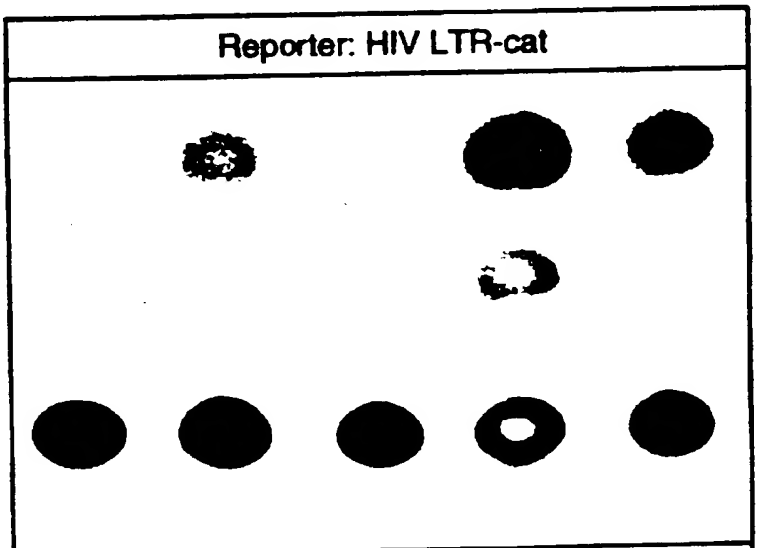

SacII 121
Xmnl 5532
5'LTR
SpeI 870
BglI 5051
pLLLgptSVtat
BstEII 1297
5929 bp
HindIII 1631
gpt
NdeI 3866
3'LTR
SV-tat
2171
XhoI
NcoI
BamHI
XbaI 3272
SalI
NruI
ClaI

FIGURE 8

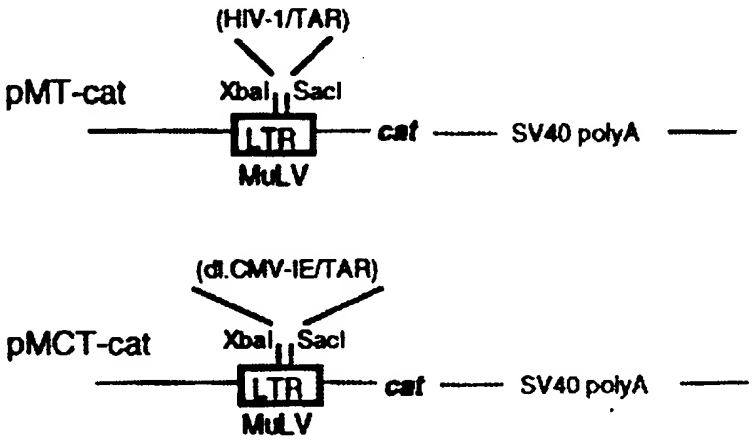

X
tat
SV40
tat
pA
MuLV LTR
gpt
SV40
tat
pA
+
cat
pA
CMV
tat
pA
RSV LTR
tat
pA
HIV-LTR

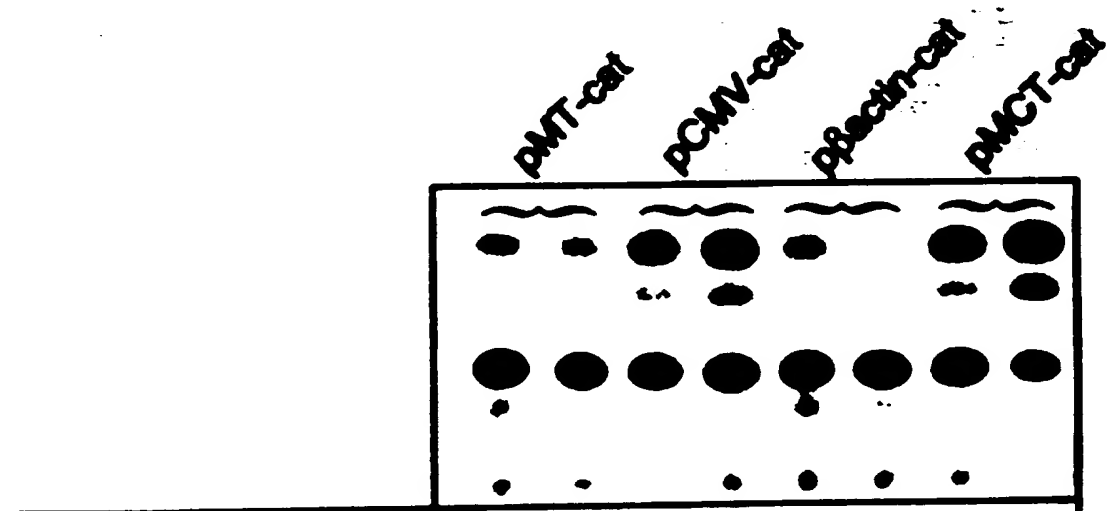


Promoter	X	SV40	MuLV/ SV40	CMV	RSV
%Conversion	0.2	10.3	5.4	66.2	28.3

FIG. 9

FIGURE 10


(HIV-1/TAR)
pMT-cat
XbaI
SacI
LTR
MuLV
cat
SV40 polyA
(dl.CMV-IE/TAR)
pMCT-cat
XbaI
SacI
LTR
MuLV
cat
SV40 polyA



TAT :	-	+	-	+	-	+	-	+
Relative CAT Expression:	1	1	12	15	1	0.7	30	140

FIG. 11

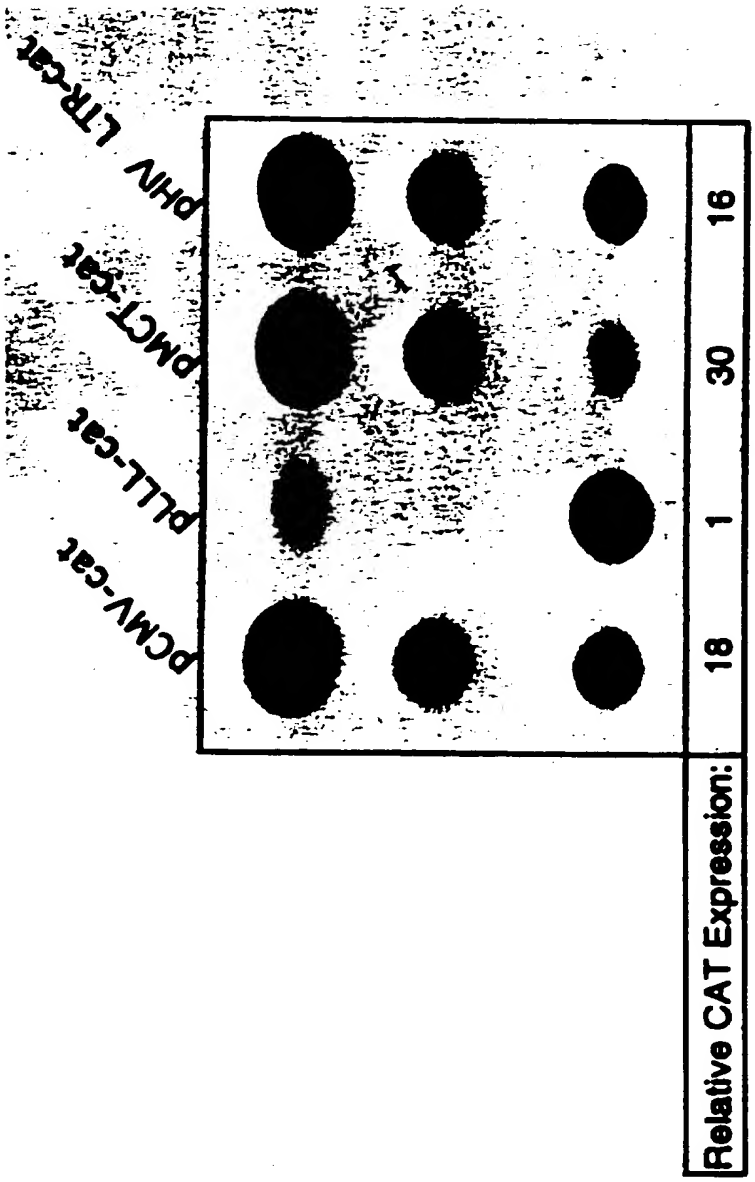

pCMV-cat
pLLL-cat
pMCT-cat
pHIV LTR-cat
Relative CAT Expression:
18
1
30
16

FIG. 12

FIG. 13

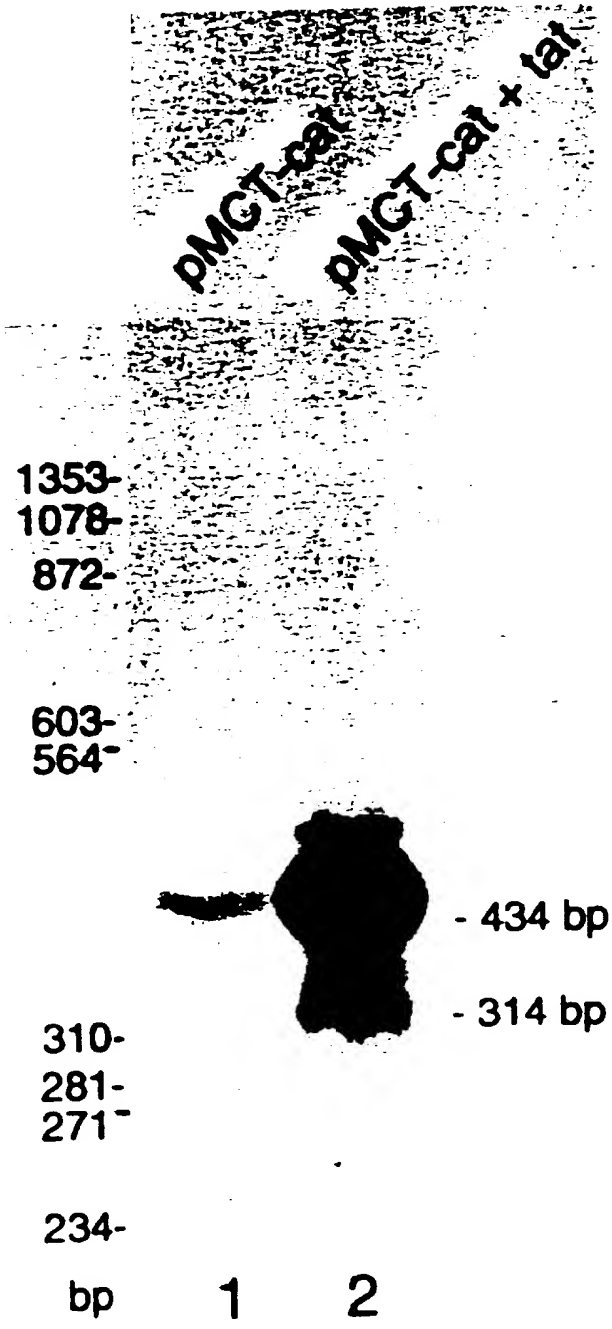

pMGT-cat
pMGT-cat + tat
1353-
1078-
872-
603-
564-
- 434 bp
- 314 bp
310-
281-
271-
234-
bp
1
2

FIG. 14

FIGURE 15

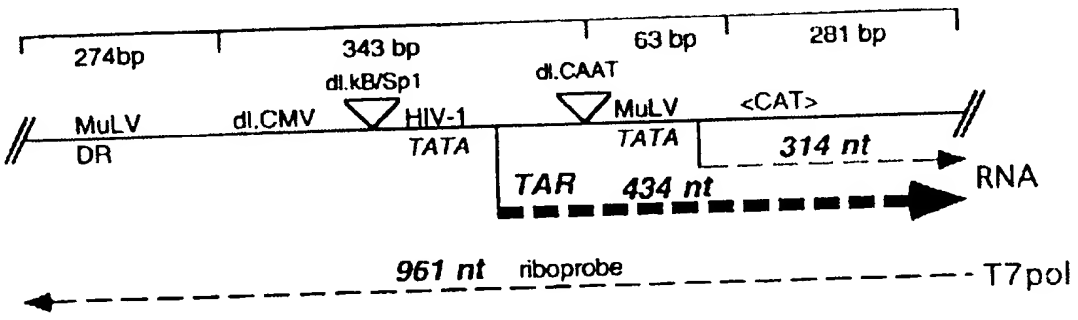
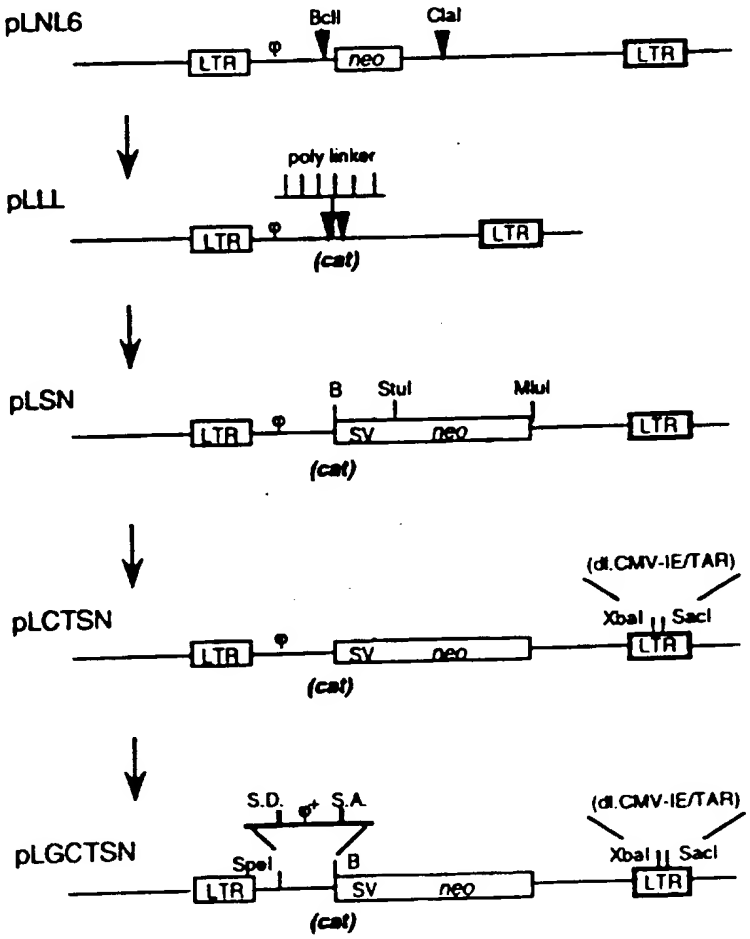

274bp
343 bp
63 bp
281 bp
dl.kB/Sp1
dl.CAAT
MuLV
DR
dl.CMV
HIV-1
TATA
MuLV
TATA
<CAT>
314 nt
RNA
TAR
434 nt
961 nt
riboprobe
T7pol

FIGURE 16


pLNL6
BclI
ClaI
LTR
φ
neo
LTR
pLLL
poly linker
LTR
φ
LTR
(cat)
pLSN
B
StuI
MluI
LTR
φ
SV
neo
LTR
(cat)
pLCTSN
(dl.CMV-IE/TAR)
XbaI
SacI
LTR
φ
SV
neo
LTR
(cat)
pLGCTSN
S.D.
φ+
S.A.
(dl.CMV-IE/TAR)
SpeI
B
XbaI
SacI
LTR
SV
neo
LTR
(cat)

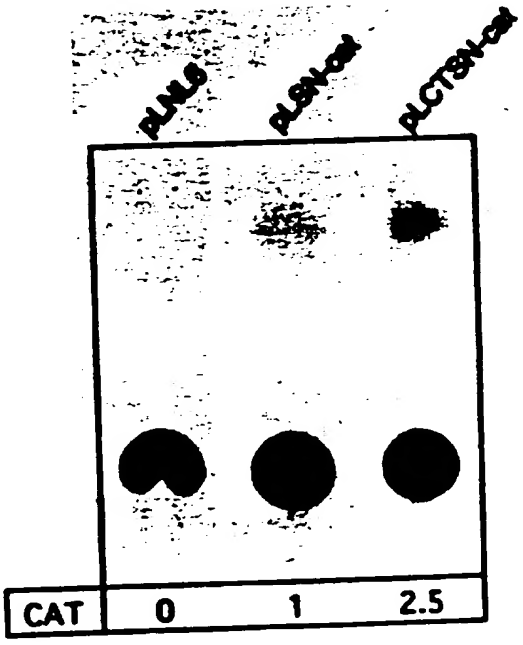


FIG. 17

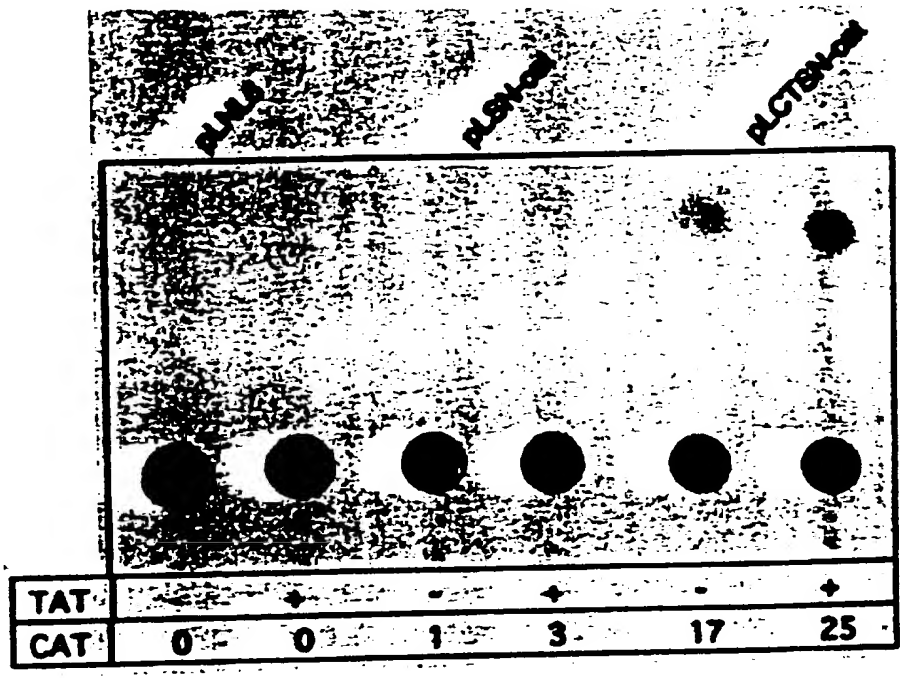


FIG. 18

FIGURE 19

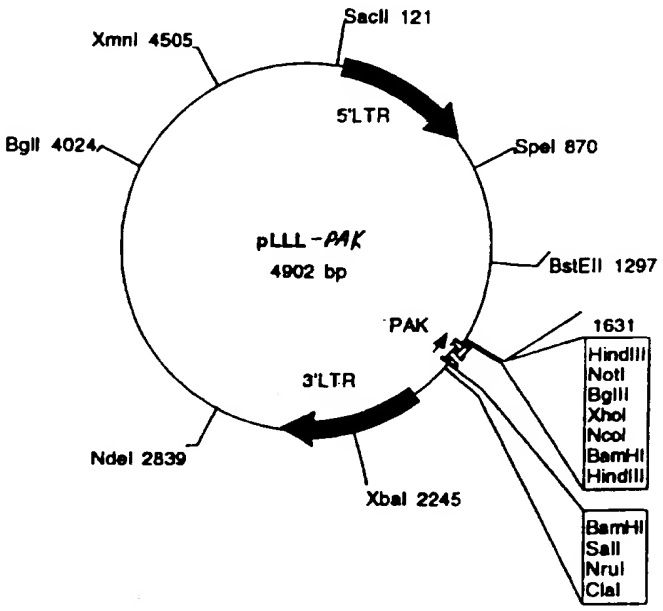

SacII 121
XmnI 4505
5'LTR
BglI 4024
SpeI 870
pLLL-PAK
4902 bp
BstEII 1297
PAK
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
HindIII
3'LTR
NdeI 2839
XbaI 2245
BamHI
SalI
NruI
ClaI

FIGURE 20

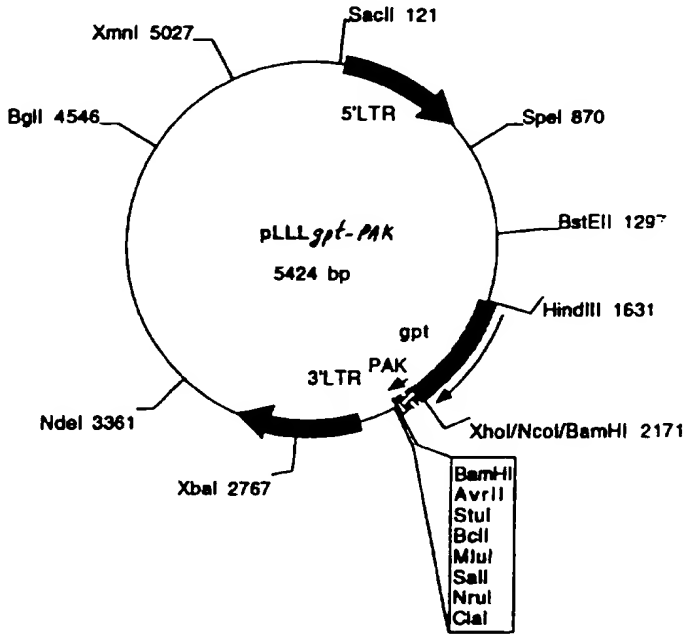

SacII 121
Xmnl 5027
5'LTR
SpeI 870
BglI 4546
pLLL gpt-PAK
5424 bp
BstEII 129
HindIII 1631
gpt
PAK
3'LTR
NdeI 3361
XhoI/NcoI/BamHI 2171
XbaI 2767
BamHI
AvrII
StuI
BclI
MluI
SalI
NruI
ClaI

FIGURE 21

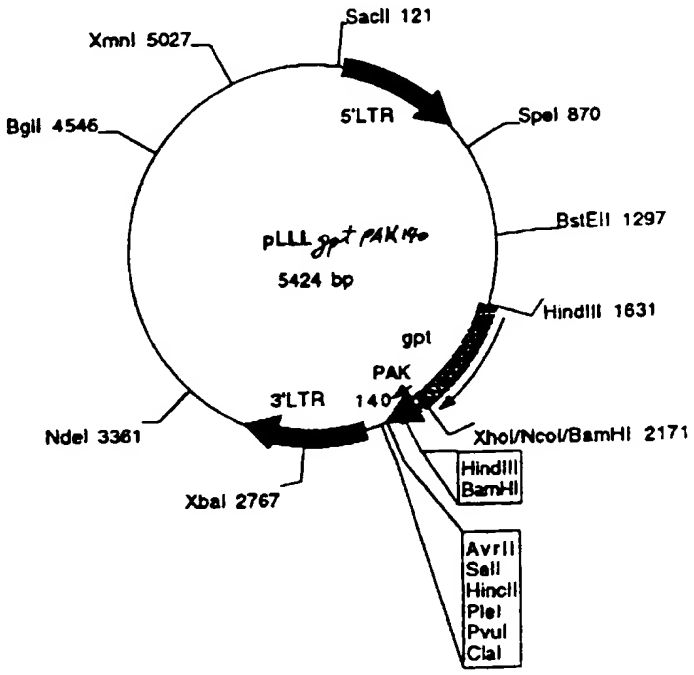

SacII 121
Xmnl 5027
5'LTR
SpeI 870
Bgll 4546
pLLL gpt PAK 140
5424 bp
BstEII 1297
HindIII 1631
gpt
PAK
140
3'LTR
NdeI 3361
XhoI/NcoI/BamHI 2171
HindIII
BamHI
XbaI 2767
AvrII
SalI
HincII
PleI
PvuI
ClaI

FIGURE 22

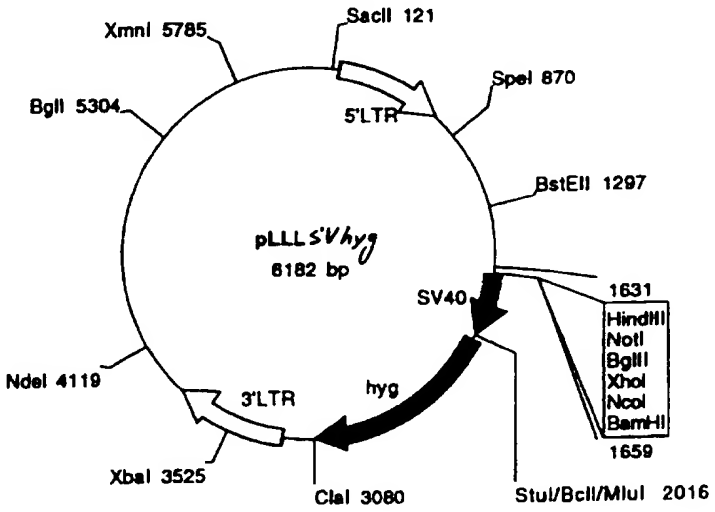
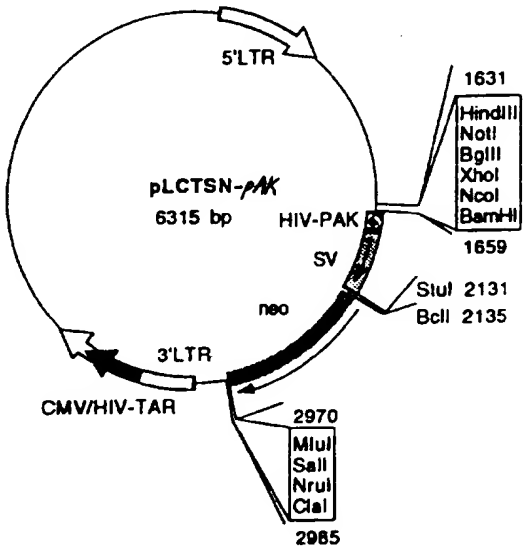

SacII 121
XmnI 5785
SpeI 870
BglI 5304
5'LTR
BstEII 1297
pLLL5'Vhyg
6182 bp
SV40
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
1659
NdeI 4119
3'LTR
hyg
XbaI 3525
ClaI 3080
StuI/BclI/MluI 2016

FIGURE 23


5'LTR
1631
HindIII
NotI
BglII
XhoI
NcoI
BamHI
1659
pLCTSN-pAK
6315 bp
HIV-PAK
SV
StuI 2131
BclI 2135
neo
3'LTR
CMV/HIV-TAR
2970
MluI
SalI
NruI
ClaI
2985

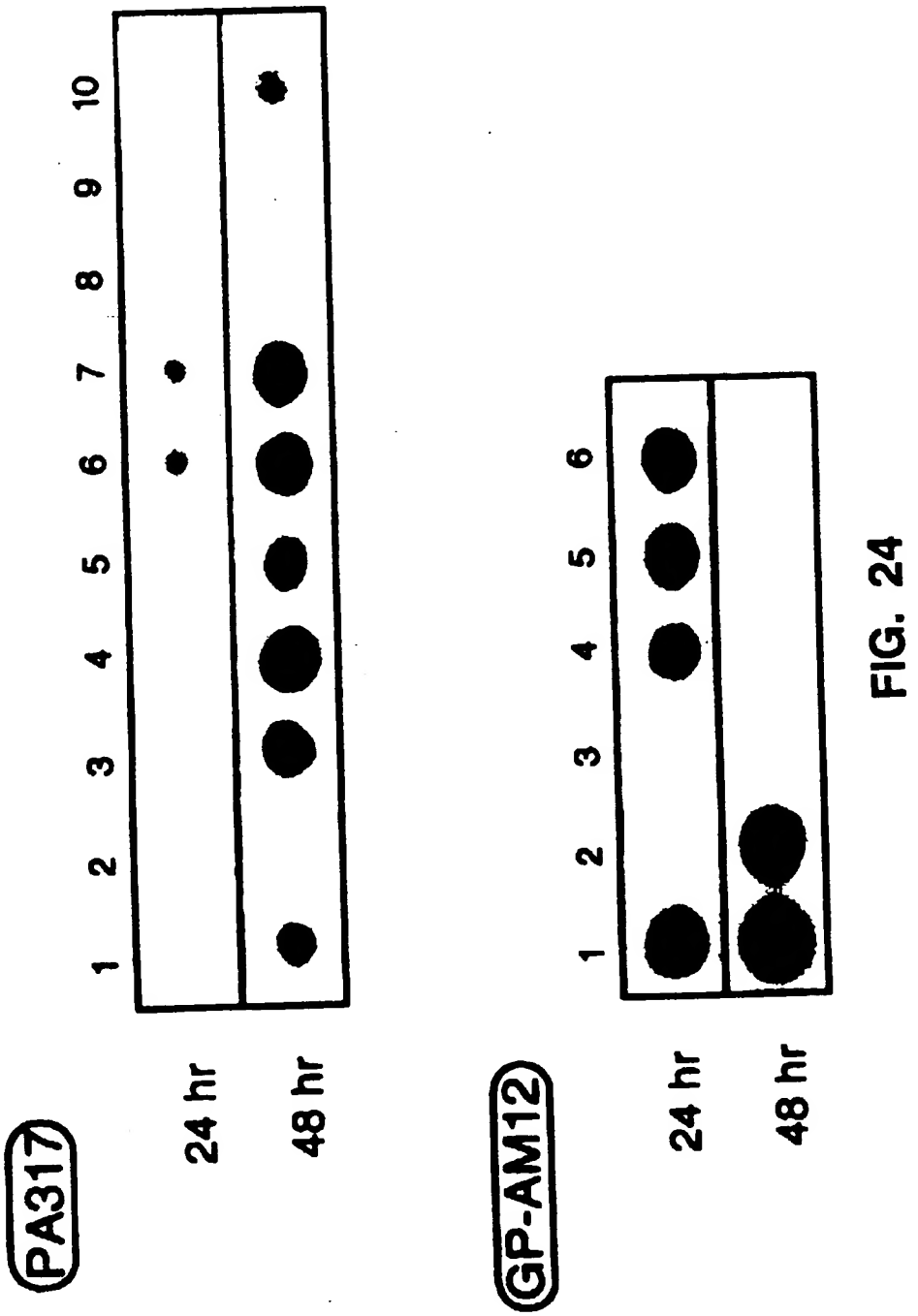


FIG. 24

Figure 25

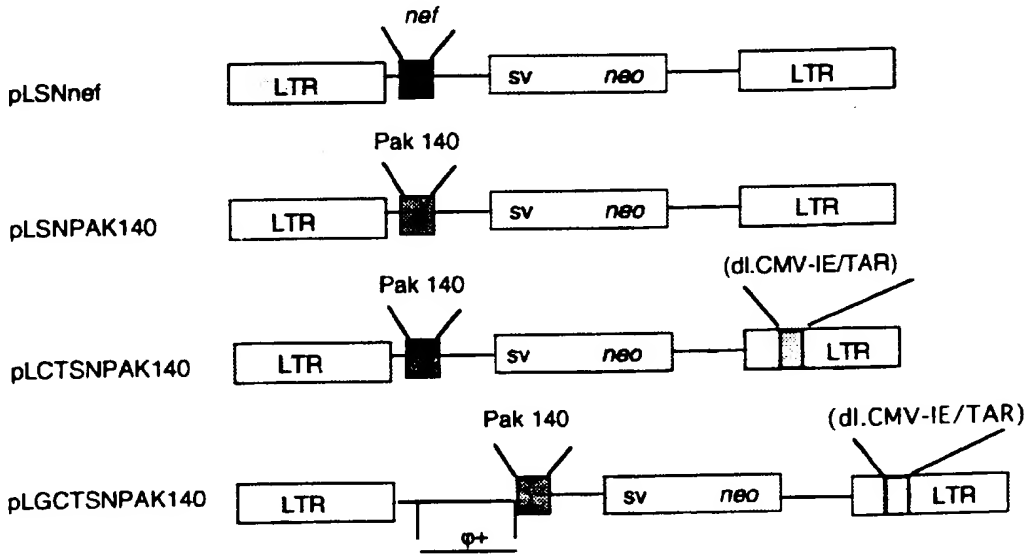

pLSNnef
nef
LTR
sv
neo
LTR
pLSNPAK140
Pak 140
LTR
sv
neo
LTR
pLCTSNPAK140
Pak 140
(dl.CMV-IE/TAR)
LTR
sv
neo
LTR
pLGCTSNPAK140
Pak 140
(dl.CMV-IE/TAR)
LTR
φ+
sv
neo
LTR

Figure 26A

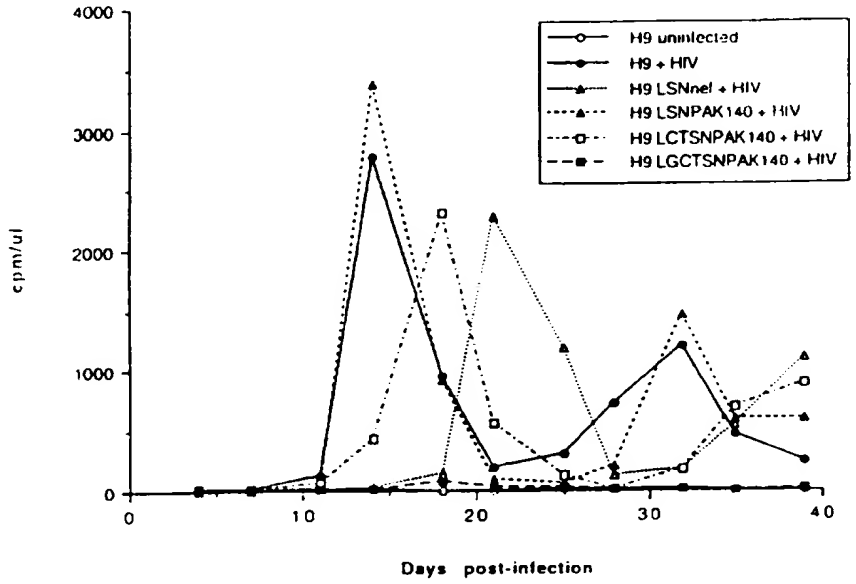


Figure 26B

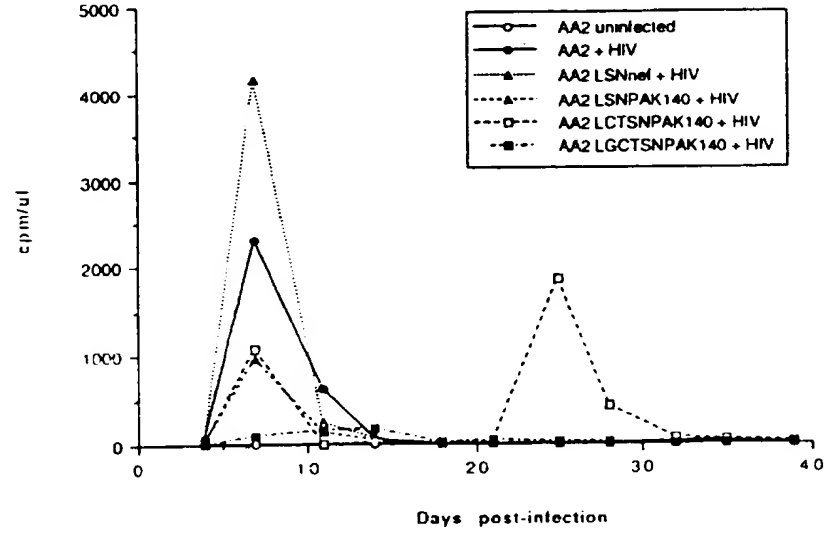


Figure 27A

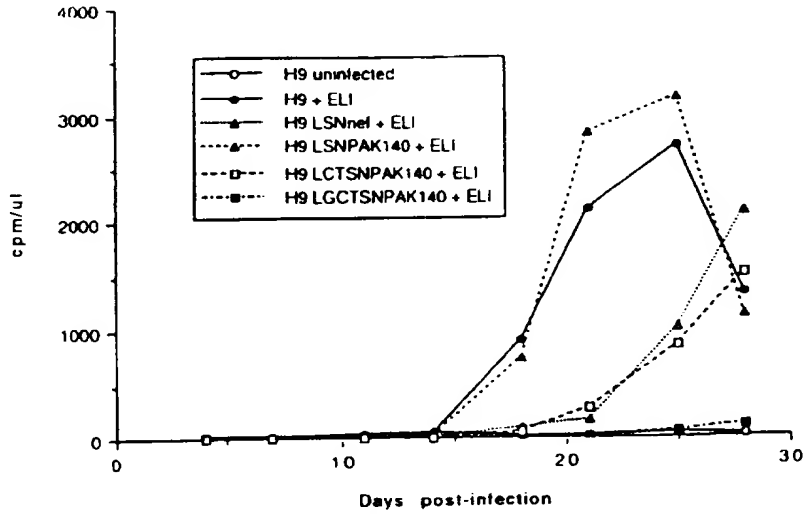
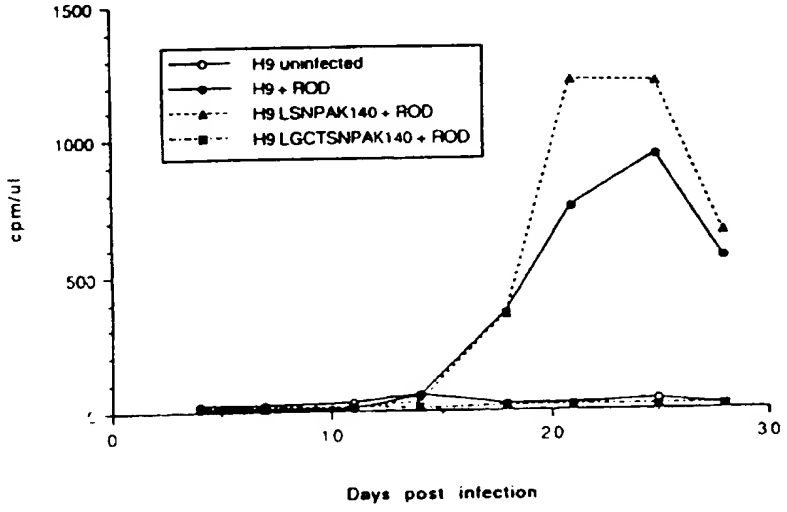


Figure 27B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14576

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/24.1; 435/91.4, 91.41, 91.42, 172.3, 252.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.1; 435/91.4, 91.41, 91.42, 172.3, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF VIROLOGY, Volume 67, Number 2, issued February 1993, Chang et al., "Human Immunodeficiency Viruses Containing Heterologous Enhancer/Promoters are Replication Competent and Exhibit Different Lymphocyte Tropisms", pages 743-752, see entire document.	1, 2, 4-15, 26
Y	JOURNAL OF VIROLOGY, Volume 61, Number 5, issued May 1987, Bender et al., "Evidence that the Packaging Signal of Moloney Murine Leukemia Virus Extends into the gag Region", pages 1639-1646, see entire document.	1, 2, 4-15, 26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
29 FEBRUARY 1996

Date of mailing of the international search report
20 MAR 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
JEFFREY S. PARKIN, PH.D.
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14576

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENE, Volume 106, Number 2, issued October 1991, Akagi et al., "Murine retroviral vectors expressing the tax1 gene of human T-cell leukemia virus type 1", pages 255-259, see entire document.	1, 2, 4-15, 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14576

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.: 3, 16-25, and 27-33
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Applicants did not comply with the sequence requirements pursuant to WIPO Standard ST.23, under Section 208, Annex C of the Administrative Instructions.

3. ☒ Claims Nos.: 28-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14576

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C12N 1/20, 15/00, 15/09, 15/63, 15/64, 15/66, 15/70, 15/74

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HARDING, Charles T.
D. YOUNG & CO.
21 New Fetter Lane
London EC4A 1DA
GRANDE BRETAGNE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	18.08.98
Applicant's or agent's file reference: PCT 506 1 CTH hlb	**REPLY DUE** within **3 month(s)** from the above date of mailing

International application no.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/02859	17/10/1997	17/10/1996

International Patent Classification (IPC) or both national classification and IPC
C12N15/86

Applicant
OXFORD BIOMEDICA (UK) LIMITED et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This report contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and / or arguments, see Rule 66.4bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 17/02/1999

Name and mailing address of the international preliminary examining authority

European Patent Office
D-80298 Munich
Tel (+49-89) 2399-0, Tx 523656 epmu d
Fax (+49-89) 2399-4465

Authorized officer / Examiner
Vollbach, S

Formalities officer (incl. extension of time limits)
Heisel, M
Telephone No (+49-89) 2399-8051

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
KP/1620		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 97/02859	17/10/1997	17/10/1996
Applicant		
OXFORD BIOMEDICA (UK) LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [X] furnished by the applicant separately from the international application,

   [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] Transcribed by this Authority

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   RETROVIRAL VECTORS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 8

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/GB 97/02859

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 15 is directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
STEVENS, HEWLETT & PERKINS
Attn. PRIVETT, Kathryn L.
1 Serjeant's Inn
Fleet Street
London EC4Y 1LL
UNITED KINGDOM

16/4(10)

Date of mailing *(day/month/year)* 17/03/1998	
Applicant's or agent's file reference KP/1620	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/GB 97/02859	International filing date *(day/month/year)* 17/10/1997
Applicant OXFORD BIOMEDICA (UK) LIMITED et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicants's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer A. Overbeeke

Form PCT/ISA/220 (January 1994)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments mustbe explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* KP/1620

Box No. I TITLE OF INVENTION

IMPROVED RETROVIRAL VECTORS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

OXFORD BIOMEDICA (UK) LIMITED
Medawar Centre
Robert Robinson Avenue
The Oxford Science Park
Oxford
OX4 4GA
UNITED KINGDOM

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KINGSMAN, SUSAN MARY
Greystones
Middle Street
Islip
Oxon
OX5 2SF
UNITED KINGDOM

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PRIVETT, KATHRYN LOUISE
Stevens Hewlett & Perkins
1 Serjeants' Inn
Fleet Street
LONDON
EC4Y 1LL
UNITED KINGDOM

Telephone No. 0171-936-2499

Facsimile No. 0171-430-2262

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. 2

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KINGSMAN, ALAN JOHN
Greystones
Middle Street
Islip
Oxon
OX5 2SF
UNITED KINGDOM

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes, at least one must be marked)*:

Regional Patent

- [X] AP **ARIPO Patent:** **GH** Ghana, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] EA **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] EP **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] OA **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] AL Albania
- [X] AM Armenia
- [X] AT Austria
- [X] AU Australia
- [X] AZ Azerbaijan
- [X] BA Bosnia and Herzegovina
- [X] BB Barbados
- [X] BG Bulgaria
- [X] BR Brazil
- [X] BY Belarus
- [X] CA Canada
- [X] CH and LI Switzerland and Liechtenstein
- [X] CN China
- [X] CU Cuba
- [X] CZ Czech Republic
- [X] DE Germany
- [X] DK Denmark
- [X] EE Estonia
- [X] ES Spain
- [X] FI Finland
- [X] GB United Kingdom
- [X] GE Georgia
- [X] GH Ghana
- [X] HU Hungary
- [X] IL Israel
- [X] IS Iceland
- [X] JP Japan
- [X] KE Kenya
- [X] KG Kyrgyzstan
- [X] KP Democratic People's Republic of Korea
- [X] KR Republic of Korea
- [X] KZ Kazakstan
- [X] LC Saint Lucia
- [X] LK Sri Lanka
- [X] LR Liberia
- [X] LS Lesotho
- [X] LT Lithuania
- [X] LU Luxembourg
- [X] LV Latvia
- [X] MD Republic of Moldova
- [X] MG Madagascar
- [X] MK The former Yugoslav Republic of Macedonia
- [X] MN Mongolia
- [X] MW Malawi
- [X] MX Mexico
- [X] NO Norway
- [X] NZ New Zealand
- [X] PL Poland
- [X] PT Portugal
- [X] RO Romania
- [X] RU Russian Federation
- [X] SD Sudan
- [X] SE Sweden
- [X] SG Singapore
- [X] SI Slovenia
- [X] SK Slovakia
- [X] SL Sierra Leone
- [X] TJ Tajikistan
- [X] TM Turkmenistan
- [X] TR Turkey
- [X] TT Trinidad and Tobago
- [X] UA Ukraine
- [X] UG Uganda
- [X] US United States of America
- [X] UZ Uzbekistan
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Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [X] ID Indonesia
- [ ]
- [ ]
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In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day month year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) GB	17 October 1996 (17-10-96)	9621679.1	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s) : (1)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / EP

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day month year):* Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1 request	:	4 sheets
2 description	:	23 sheets
3 claims	:	2 sheets
4. abstract	:	1 sheets
5 drawings	:	7 sheets
Total	:	37 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☒ other *(specify)*: Form 23/77

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

PRIVETT, KATHRYN LOUISE

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
PCT 506 1 CTH hlb		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/02859	17/10/1997	17/10/1996
International Patent Classification (IPC) or national classification and IPC		
C12N15/86		
Applicant		
OXFORD BIOMEDICA (UK) LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/05/1998	25.01.99
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0. Tx 523656 epmu d Fax: (+49-89) 2399-4465	Vollbach, S Telephone No. (+49-89) 2399-8715

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HARDING, Charles T.
D. YOUNG & CO.
21 New Fetter Lane
London EC4A 1DA
GRANDE BRETAGNE

CTH

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)* 25. 01. 99	
Applicant's or agent's file reference PCT 506 1 CTH hlb	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/02859	17/10/1997	17/10/1996

Applicant
OXFORD BIOMEDICA (UK) LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx 523656 epmu d Fax: (+49-89) 2399-4465	Houyez-Stevens, M Tel. (+49-89) 2399-8163

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
PCT 506 1 CTH hlb		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/02859	17/10/1997	17/10/1996
International Patent Classification (IPC) or national classification and IPC		
C12N15/86		
Applicant		
OXFORD BIOMEDICA (UK) LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/05/1998	25. 01. 99
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Vollbach, S Telephone No. (+49-89) 2399-8715

Form PCT/IPEA/409 (Cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB97/02859

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-23 as originally filed

Claims, No.:

1-16 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. The present application relates to retroviral vectors, which are improved over the known vectors such as e.g. the vector system denominated TIN vectors. This vector system is characterized by the inducibility of tat. A disadvantage of said system consists in the low basal level of transcription from the HIV LTR promoter. Thus the underlying technical problem of the present application consists in the provision of a vector system which have nearly no basal transcription anymore. The solution to said problem consists in the finding that an additional regulatory element derivable from HIV namely the RRE is incorporated into the vector rendering expression dependent upon Rev. Important, however, is the finding that the desired result (i.e. no basal transcription) is obtained only when the vector contains in addition to the RRE also extended sequences derived from the HIV env region and the 3' splice acceptor sequence from env. With the vector thus produced, no basal transcription was detectable in the absence of Tat and Rev.

2. Although various documents are available which concern retroviral vectors, the cited prior art does not disclose or suggest the vector described in the description of the present application.
Nevertheless the following objections apply to present set of claims.
In view of the fact that retroviral vectors containing one or more of the functional elements of present vector system are known in the art (see e.g. D1 = WO92/21750 as well as the documents cited in the description of the present application), the present authority takes the opinion that claim 1 in the present form does not contain all essential features in order to solve the above defined technical problem. Therefore, the essential features which are missing are that the fragment containing the RRE also contains the 3 'splice acceptor sequence from the HIV env and additionally that the vector is a non-HIV based vector (the latter feature is important with regard to all know HIV based vector systems, which always contain the splice sequence and the RRE). In general, an embodiment defined in the application to be a preferred embodiment, can in the light of the prior art become an essential feature to the invention. This exactly apply in the present case. Therefore, the introduction of the above mentioned features is necessary in order to delimit the present claims in an inventive manner from the prior art.

3. Thus claims amended in the proposed way are allowable under Article 33(2) and Article 33(3) PCT. However, the claims in the present form are inadmissible under

Article 33(3) PCT as well as under Article 6 PCT because essential technical features are missing in said claims.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HARDING, Charles, Thomas
D Young & Co
21 New Fetter Lane
London EC4A 1DA
ROYAUME-UNI

Date of mailing (day/month/year)
16 June 1998 (16.06.98)

Applicant's or agent's file reference
KP/1620

IMPORTANT NOTIFICATION

International application No.
PCT/GB97/02859

International filing date (day/month/year)
17 October 1997 (17.10.97)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
PRIVETT, Kathryn, Louise
Stevens Hewlett & Perkins
1 Serjeants' Inn
Fleet Street
London EC4Y 1LL
United Kingdom

State of Nationality:
State of Residence:
Telephone No.: 0171-936-2499
Facsimile No.: 0171-430-2262
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
HARDING, Charles, Thomas
D Young & Co
21 New Fetter Lane
London EC4A 1DA
United Kingdom

State of Nationality:
State of Residence:
Telephone No.: 44-1703-634816
Facsimile No.: 44-1703-224262
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[X] other: PRIVETT, Kathryn, Louise

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Nicola Wolff

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

002085434

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 16 June 1998 (16.06.98)	
International application No. PCT/GB97/02859	**Applicant's or agent's file reference** KP/1620
International filing date (day/month/year) 17 October 1997 (17.10.97)	**Priority date** (day/month/year) 17 October 1996 (17.10.96)
Applicant KINGSMAN, Susan, Mary et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 May 1998 (15.05.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nicola Wolff
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

2085430

1. The present application relates to retroviral vectors, which are improved over the known vectors such as e.g. the vector system denominated TIN vectors. This vector system is characterized by the inducibility of tat. A disadvantage of said system consists in the low basal level of transcription from the HIV LTR promoter. Thus the underlying technical problem of the present application consists in the provision of a vector system which have nearly no basal transcription anymore. The solution to said problem consists in the finding that an additional regulatory element derivable from HIV namely the RRE is incorporated into the vector rendering expression dependent upon Rev. Important, however, is the finding that the desired result (i.e. no basal transcription) is obtained only when the vector contains in addition to the RRE also extended sequences derived from the HIV env region and the 3' splice acceptor sequence from env. With the vector thus produced, no basal transcription was detectable in the absence of Tat and Rev.

2. Although various documents are available which concern retroviral vectors, the cited prior art does not disclose or suggest the vector described in the description of the present application.
Nevertheless the following objections apply to present set of claims.
In view of the fact that retroviral vectors containing one or more of the functional elements of present vector system are known in the art (see e.g. D1 = WO92/21750 as well as the documents cited in the description of the present application), the present authority takes the opinion that claim 1 in the present form does not contain all essential features in order to solve the above defined technical problem. Therefore, the essential features which are missing are that the fragment containing the RRE also contains the 3 'splice acceptor sequence from the HIV env and additionally that the vector is a non-HIV based vector (the latter feature is important with regard to all know HIV based vector systems, which always contain the splice sequence and the RRE).

3. Thus claims which were amended in the proposed way are allowable under Article 33(2) and Article 33(3) PCT. However, the claims in the present form are inadmissible under Article 33(3) PCT as well as under Article 6 PCT because essential technical features are missing in said claims.

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Correspondence with the EPO on PCT Chapter II demands

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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 5/10, 7/00, A61K 48/00, C07K 14/16

A1

(11) International Publication Number: **WO 98/17817**

(43) International Publication Date: 30 April 1998 (30.04.98)

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(22) International Filing Date: 17 October 1997 (17.10.97)

(30) Priority Data:
9621679.1 17 October 1996 (17.10.96) GB

(71) Applicant *(for all designated States except US)*: OXFORD BIOMEDICA (UK) LIMITED [GB/GB]; Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KINGSMAN, Susan, Mary [GB/GB]; Greystones, Middle Street, Islip, Oxon OX5 2SF (GB). KINGSMAN, Alan, John [GB/GB]; Greystones, Middle Street, Islip, Oxon OX5 2SF (GB).

(74) Agent: PRIVETT, Kathryn, Louise; Stevens Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

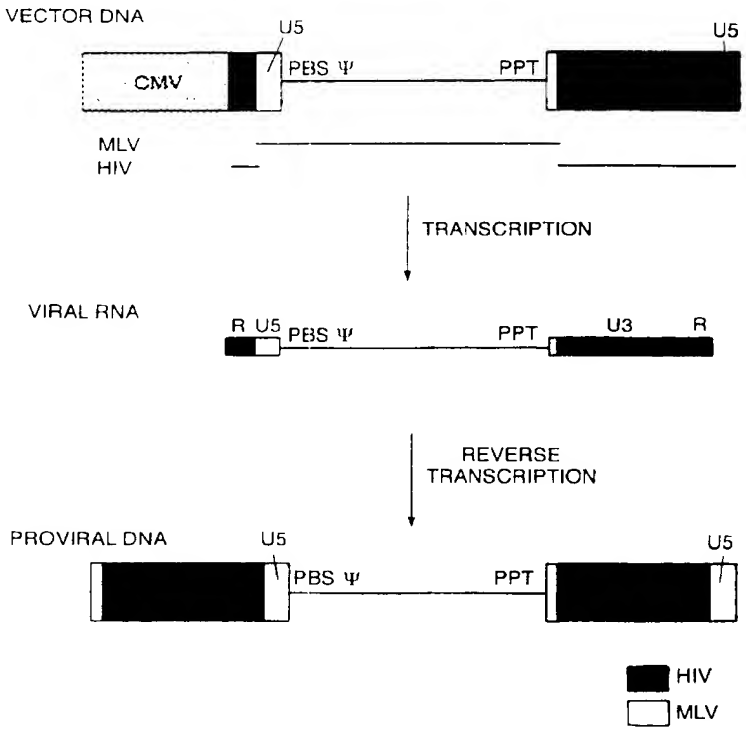
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RETROVIRAL VECTORS

(57) Abstract

Retroviral vector particles having an RNA genome carrying sequences which provide in the DNA provirus at least one selected gene located within an intron in a transcription unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor such as HIV Rev. Expression of the selected genes is thus rendered Rev-dependent and so is dependent upon the presence of HIV.



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RETROVIRAL VECTORS

This invention relates to retroviral vector particles and to DNA constructs encoding RNA genomes for retroviral vectors. In particular it relates to retroviral vectors for gene therapy for treatment or prevention of retrovirus infections, such as HIV.

Therapeutic molecules for use in HIV gene therapy include ribozymes, trans-dominant proteins, scFv molecules, antisense constructs and TAR and RRE decoys (reviewed in Yu *et al.* 1994). These molecules are envisaged to act both as therapy against already infected cells and as protective 'intracellular immunisation' (Baltimore, 1988) in uninfected cells. In addition, the use of toxins (suicide genes) or immunological markers has also been proposed as a means of killing infected cells and so reducing the viral load in the patient.

For many of these molecules, it is desirable that expression can be regulated. This is clearly the case for suicide genes, but the constitutive expression of a therapeutic protein may also be undesirable in that it may cause some cellular toxicity or lead to a host immunological response. Furthermore, toxic side effects are also possible from the expression of RNA molecules in cells, including the inappropriate induction of interferon responses. In the case of HIV, the viral LTR promoter itself has the characteristic of being an inducible promoter, directing low basal levels of transcription in the absence of the virally encoded Tat trans-activator protein (Arya *et al.* 1985). Tat-inducibility is a property of sequences in the U3 region of the LTR and the TAR sequence in the R region (Berkhout and Jeang, 1992). Allowing the HIV LTR to direct expression of a therapeutic gene will therefore limit its expression to those cells infected by HIV. Several groups have already started exploring ways of using this property of the LTR and have demonstrated inducible expression of genes upon HIV infection (Caruso *et al.* 1992, Brady *et al.*

1994). Recently we have developed a novel vector system, the TIN system, that has clear advantages for HIV gene therapy (PCT/GB96/01230). This system comprises novel MLV based Tat-inducible (TIN) vectors, designed for the 5' positioning of a Tat induced therapeutic gene (TITG), and incorporating features to allow the efficient packaging, reverse transcription and integration of the vector genome by the MLV machinery. To facilitate the production of high levels of vector genomes in the producer cells (standard MLV packaging lines or our transient transfection system (Soneoka *et al.* 1995)), the packagable vector transcript is preferably driven from the CMV promoter rather than the natural vector MLV LTR, A clear advantage of this system is the achievement of HIV control of gene expression from a simple transcription unit which can be transduced by the standard MLV based vector technology. The TIN vector system was proposed to have additional advantages in that the low basal levels of transcription from the HIV LTR promoter would reduce problems of interference with any downstream constitutive promoters, facilitating stable expression of any constitutive gene from the vector in the transduced cells.

Where the therapeutic gene is a non natural protein there always exists the possibility of immune recognition and destruction of the cell expressing this protein. This is clearly undesirable if the aim is to create a reservoir of HIV resistant cells in the patient. It is therefore important to have basal levels of expression as close to zero as possible. The data using TIN vectors that we have previously described clearly demonstrate very low basal levels of expression but the levels were still detectable. Furthermore it is known that in some target cells additional cellular factors may be present that enhance the basal transcription from the HIV promoter in the absence of Tat. Indeed some transcription from the HIV LTR must occur to produce Tat itself. In the T cell population exposure to cytokines, antigens and various stress factors such as hypoxia

or UV irradiation are all known to stimulate transcription from the HIV LTR (Valerie *et al* 1988; Nabel & Baltimore 1987). Given that these types of stimuli are characteristic features of immune responses there therefore exists the possibility that basal expression from TIN vectors could be elevated under certain conditions within an HIV infected patient. It is therefore desirable to provide additional controls on the basal levels of expression.

There is an additional regulatory circuit in HIV gene expression which involves the virally encoded Rev protein and its target RNA sequence the Rev response element (RRE) (reviewed by Cullen 1995 and explored by Naldini *et al* 1996). HIV has two introns in its genome and regulates splicing to generate unspliced RNA genomes and two classes of sub-genomic mRNA. To ensure that some RNA remains unspliced and is exported to the cytoplasm for incorporation into new virus particles the second intron contains a specific recognition sequence for a virally encoded protein called Rev. This RNA sequence is the Rev response element (RRE), for which a minimal functional sequence of 270 nucleotides has been identified (Huang *et al* 1991). Rev is a 13kD protein that specifically binds to RRE and activates the export of RRE-containing RNA (Malim *et al* 1989b). It appears that the HIV introns are recognised by the cellular splice commitment factors; but the splicing process is inefficient which leads to entrapment of the RNAs within the nucleus. The Rev/RRE interaction bypasses this entrapment and exports the RNA to the cytoplasm. If splicing is made more efficient then the RNA export becomes Rev independent (Chang and Sharp 1989; Hammarskjold *et al* 1994). If however gene expression is made more efficient then unspliced RNA can appear in the cytoplasm (e.g. D'Agostino *et al* 1992). In addition certain sequence elements referred to as cis inhibitory sequences (CRSs) might contribute to the Rev dependency of HIV by promoting nuclear entrapment and/or RNA instability (e.g. Cochrane *et al* 1991). There is therefore an

interaction between splicing efficiency, expression efficiency and CRSs that may influence Rev dependency. The interaction is not necessarily predictable or definable and may vary in different cell types (as discussed by Cullen 1995). The mechanistic details of Rev-mediated export have not yet been established but cellular export proteins are involved (e.g. Fritz *et al* 1995).

One potential approach to achieving additional regulation of a retroviral vector for HIV gene therapy would therefore be to incorporate RRE into the vector rendering expression dependent upon Rev. Such an approach may be obvious for vectors specifically based upon HIV itself as these will of necessity contain the Rev/RRE system, unless removed. For example Naldini *et al* (1996) use a fragment of HIV genome encompassing the normal major splice donor sequence, RRE and the normal splice acceptor sequence in the normal configuration in their transducing vector pHR'. RRE is therefore contained within the HIV env intron. This fragment is presumed and is likely, but not proven, to render the expression of the genome responsive to Rev. In the vector described in this paper the coding sequence lies outside of the RRE containing intron.

A non-lentiviral retroviral vector incorporating the Rev/RRE system has been described by Lisziewicz (US patent publication PB92-139336) whereby the RRE element is inserted into a retroviral vector or into the intron of a foreign gene contained within that vector. The constructions outlined make no reference to the presence of the splice donor sequence within the MLV vector, nor take into account any requirement for inefficient splicing to achieve Rev function, and there is no description of precisely where to insert the RRE. The disclosure therefore suggests that in constructing a retroviral vector whose expression is dependent upon Rev the nature and location of the RRE is not material and the nature and location of additional introns or splice sequences is not material.

We now show that a TIN vector can be converted into a Rev responsive vector by the insertion of RRE to create TRIN (Tat and Rev inducible) vectors. However, contrary to the finding in Lisziewicz, we demonstrate that simply inserting RRE into a TIN vector does not achieve a strict dependency on Rev and that there is therefore no significant advantage in this construction with respect to reducing basal gene expression beyond that obtained with TIN vectors. Moreover, we show that the inclusion of an extended sequence derived from the HIV env region which contains RRE and the 3' splice acceptor sequence from env produces a new vector pTRAC for which the basal transcription levels are undetectable in the absence of Tat and Rev. We propose that this undetectable expression is due to the combination of nuclear retention of the RNA via recognition of a hybrid MLV/HIV intron and the fact that any RNA that is spliced rather than exported via the Rev system will have the reporter/therapeutic gene removed during splicing.

The invention therefore provides in one aspect a retroviral vector particle comprising a packagable RNA genome capable of being inserted into a target cell genome when in the form of a DNA provirus, said RNA genome carrying sequences which provide in the DNA provirus at least one selected gene capable of being expressed in the target cell and located within an intron in a transcription unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor.

In another aspect, the invention provides a DNA construct encoding the packagable RNA genome for the retroviral vector particle described herein, under the control of a promoter. The selected gene or genes may be present in or absent from the DNA construct. If they are absent, the DNA construct has an insertion site e.g. a unique restriction enzyme site at which the selected gene or genes may be inserted, the site

located within the intron such that it is flanked by a splice donor and a splice acceptor sequence.

In a further aspect, the invention provides a retroviral vector particle production system comprising a host cell transfected with a DNA construct as described herein, said system capable of producing retroviral vector particles as described herein. The host cell may be a packaging cell line or it may be a suitable host cell transfected with nucleic acid sequences, present e.g. on plasmids, encoding the structural elements of the retroviral particles. The transfected host cell is also referred to as a producer cell.

In yet another aspect, the invention provides a retroviral vector particle production system comprising a set of nucleic acid sequences encoding the components of a retroviral vector particle as described herein.

In a still further aspect, the invention provides the use of retroviral vector particles as described herein for gene therapy, and infected or transduced target cells resulting from such use.

The retroviral vector particle according to the invention therefore provides a means for inserting into a target cell a therapeutic gene whose expression in the target cell is dependent upon the presence of a factor which enables the transport of an RNA transcript containing the transcribed gene, into the cytoplasm.

The response element in the retroviral vector genome is chosen according to the specific conditions under which expression of the therapeutic gene is desired. If expression is to be HIV dependent, then a suitable response element is RRE or a functional equivalent which responds to Rev. A functional equivalent of RRE may be for example a portion of RRE or a mutated or otherwise manipulated version of RRE which retains the desired activity.

The transport factor may thus be HIV Rev, rendering expression of the therapeutic gene dependent upon the presence of HIV. The transport factor may alternatively be any factor, originating e.g. from other viruses or from host cells, which is analogous to Rev in that it enables transport to the cytoplasm via a specific interaction with the response element. Systems analogous to the Rev/RRE system are known, for example in other retroviruses. One such example is the rex/RxRE system in HTLV-1.

In order that RNA transcripts containing the transcribed therapeutic gene may be transported to the cytoplasm in the presence of the appropriate transport factor, the intron containing the therapeutic gene needs to be an inefficiently spliced intron. This may be achieved in a variety of ways. The preferred method described herein for achieving a Rev-dependent intron in an MLV-based vector is to use the MLV splice donor site and the HIV envelope gene 3' splice site. It is within the ability of those skilled in the art to devise other suitable splice site or intron sequence combinations for HIV Rev-dependency.

Preferably, the RRE or other response element is located within the intron, to ensure Rev- (or other transport factor) dependent expression. Conveniently, RRE and the 3' splice acceptor site may be provided in a sequence derived from the HIV env region.

Advantageously, the genome of the retroviral vector particle according to the invention will have its packaging site located within the intron containing the therapeutic gene. This means that RNA that is unspliced will be trapped in the nucleus and therefore unavailable for packaging, and RNA that is spliced will have the packaging site deleted and will also be unavailable for packaging. This feature contributes to the safety of the vector system, and is present in the preferred vector configuration described herein.

The retroviral vector particle may be MLV-based. MLV systems are so far the most widely used retroviral vector systems and have been used in human gene therapy applications. Other retroviruses may be used instead however, including other oncoretroviruses (the sub-group of retroviruses containing MLV), and lentiviruses (the sub-group of retroviruses containing HIV). Examples include ASLV, SNV and RSV all of which have been split into packaging and vector components for retroviral vector particle production systems. The retroviral vector particle according to the invention may be based on a genetically or otherwise (e.g. by specific choice of packaging cell system) altered version of a particular retrovirus.

That the vector particle according to the invention is "based on" a particular retrovirus means that the vector is derived from that particular retrovirus. The genome of the vector particle comprises components from that retrovirus as a backbone. The vector particle contains essential vector components compatible with the RNA genome, including reverse transcription and integration systems. Usually these will include gag and pol proteins derived from the particular retrovirus. Thus, the majority of the structural components of the vector particle will normally be derived from that retrovirus, although they may have been altered genetically or otherwise so as to provide desired useful properties. However, certain structural components and in particular the env proteins, may originate from a different virus. The vector host range and cell types infected or transduced can be altered by using different env genes in the vector particle production system to give the vector particle a different specificity.

Preferably, the retrovirus vector genome contains the minimum retroviral material necessary to function. This is important from a safety aspect; possible reconstruction of infectious virus particles must be avoided. In any case, the retroviral vector will be replication defective.

Also, by avoiding expression of unwanted virus proteins in the target cell, undesirable immune responses are reduced. Gag-pol and env are therefore supplied in trans in the vector particle production system.

However the retrovirus vector genome clearly needs to contain the elements required for obtaining sufficiently high viral titres of vector particles from the producer cell, and for allowing it to integrate into the target cell genome. It will be evident that in order to function as a vector the retroviral vector particle according to the invention will need to have a reverse transcription system (compatible reverse transcription and primer binding sites) and an integration system (compatible integrase and integration sites) allowing conversion to the provirus and integration of the double-stranded DNA into the host cell genome. Additionally, the vector genome will need to contain a packaging signal.

In a vector particle according to the invention which is designed for use in gene therapy against a particular retrovirus, there is preferably also a control at the transcriptional level which requires the presence of that retrovirus in order to initiate or upregulate transcription of the therapeutic gene or genes. For anti-HIV gene therapy, the vector genome is preferably constructed such that in the DNA provirus the 5' LTR comprises HIV sequences sufficient to render the therapeutic gene Tat-inducible, and the therapeutic gene is located between the LTRs, yet the vector is based on a simple retroviral vector such as MLV. The therapeutic gene is under the transcriptional control of the promoter in the 5' LTR but not otherwise operably linked to any other promoter from the vector genome. This is the principle behind the TIN vectors discussed above.

In more detail, TIN vectors are constructed with the following points in mind. Tat acts on the TAR region of R, but also requires sequences in the U3 region to function properly. Certain sections of both U3 and R can be removed while still leaving an effective Tat-responsive element. Deletions in HIV-2 U3 result in promoters with lower basal levels

of transcription that still remain responsive to Tat (Brady *et al* 1990). Thus, the transcriptional control is preferably provided by an HIV Tat-inducible promoter comprising the functional portions of both U3 and R from HIV. Alternatively certain U3 and/or R sequences from other retroviruses, which are responsive to the HIV Tat protein, may be used, for example U3 from RSV linked to R from HIV. The TIN vector provides a means of preserving the Tat response within the context of a simple retroviral vector e.g. one based on MLV such that MLV packaging systems may be used.

The selected gene located in the intron of the vector genome according to the invention is preferably a therapeutic gene, that is it encodes a gene product which is active against infection or disease. Where the vector particle is for use in anti-HIV gene therapy, the product of the therapeutic gene will have an appropriate activity for that purpose. Therapeutic genes may encode for example an anti-sense RNA, a ribozyme, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen that induces antibodies or helper T-cells or cytotoxic T-cells, a single chain antibody or a tumour suppressor protein.

Where two or more genes are present in the same transcription unit, there may be an internal ribosome entry site (IRES) e.g. from picornaviral RNA, to allow both genes to be translated from a single transcript. Retroviruses incorporating IRES sequences have been constructed by others.

It will be evident that the term "gene" is used loosely here, and includes any nucleic acid coding for the desired polypeptide. Usually, the genes delivered by the vector particle according to the invention will be cDNAs.

A further gene may also be present outside the intron containing the therapeutic gene and under the control of a separate promoter. This further gene may encode for example a selectable marker, or a further therapeutic agent which may be among the therapeutic agents

listed above. Expression of this gene may be constitutive; in the case of a selectable marker this may be useful for selecting successfully transfected packaging cells, or packaging cells which are producing particularly high titers of the retroviral vector. Alternatively or additionally, the selectable marker may be useful for selecting cells which have been successfully infected with the retroviral vector and have the provirus integrated into their own genome.

One way of performing gene therapy is to extract cells from a patient, infect the extracted cells with a retroviral vector and reintroduce the cells back into the patient. A selectable marker may be used to provide a means for enriching for infected or transduced cells or positively selecting for only those cells which have been infected or transduced, before reintroducing the cells into the patient. This procedure may increase the chances of success of the therapy. Selectable markers may be for instance drug resistance genes, metabolic enzyme genes, or any other selectable markers known in the art.

However, it will be evident that for many gene therapy applications of retroviral vectors, selection for expression of a marker gene may not be possible or necessary. Indeed expression of a selection marker, while convenient for *in vitro* studies, could be deleterious *in vivo* because of the inappropriate induction of cytotoxic T lymphocytes (CTLs) directed against the foreign marker protein. Also, it is possible that for *in vivo* applications, vectors without any internal promoters will be preferable. The presence of internal promoters can affect for example the transduction titres obtainable from a packaging cell line and the stability of the integrated vector.

The DNA construct according to the invention which encodes the packagable RNA genome preferably comprises a promoter originating from a source other than the first or second retrovirus. Particularly preferred are strong promoters such as the CMV promoter which give rise

to a high level of expression of the vector RNA in the producer cell line.

The invention will now be further described with reference to the accompanying drawings in which:

Figure 1 shows a schematic outline of constructs according to the invention. The vector genome in the producer cell is shown (i). In the producer cell Rev is provided either by cotransfection or in a stable cell line by procedures known to those ordinarily skilled. The resulting vector derived RNA (ii) is unspliced and is exported to the cytoplasm by the Rev protein. In the target cell the vector genome is as in (i) but the only RNA that appears in the cytoplasm has the structure shown in (iii) because Rev is absent. Upon HIV infection when Rev is produced by the infecting virus then the retroviral vector derived RNA that reaches the cytoplasm will have the structure shown in (iv) i.e. identical to that in the producer cell (ii);

Figure 2 shows vectors used in the construction of a universal TIN vector (pTIN511);

Figure 3 shows construction of an RRE splice acceptor cassette (RAC);

Figure 4 shows construction of pTRAC;

Figure 5 shows construction of pTRAC-TG;

Figure 6 shows construction of pTRIN-TG; and

Figure 7 shows the prototype TIN vector pTIN414.

Figure 8 shows the principle of TIN vectors. The specific example of an MLV-based Tat-inducible vector is given. The packagable RNA genome comprises from the 5'end at least the functional part of the R region of HIV, all or a functional part of the MLV U5 region, a functional MLV primer binding site for first strand reverse transcription, a functional MLV packaging site, an insertion site for insertion of one or more therapeutic genes (into the DNA copy), a functional MLV primer binding site for second strand reverse transcription, a short (e.g. 10 - 100 nucleotides) sequence recognised by the MLV integration system, all or a

substantial part of the HIV U3 region, and an R region corresponding to the R region at the 5'end. Vector and proviral DNA are also shown.

Preparation of the constructs is described in detail in the Examples which follow.

This is the first description of the incorporation of an extended HIV-1 RRE element combined with an HIV-1 splice acceptor site into an MLV vector. It is the first description of a retroviral vector where the therapeutic gene is contained within an intron. The invention is particularly useful when combined with a TIN vector. That vector could be a single transcription unit vector or could contain a second transcription unit as described for pTIN501. The retroviral vector could be based on any retrovirus including lenti-viruses. In the case of lentiviral vectors a vector has been described that contains the HIV-1 RRE and the major splice site but the therapeutic gene is not included within an intron and the Rev/RRE system is not used to manipulate the expression of the therapeutic gene (Naldini *et al* I996). The components of the intron in the present invention are preferably derived from MLV and HIV.

COMPONENTS OF MLV BASED TAT-INDUCIBLE (TIN) VECTORS

We have constructed a series of vectors that are packagable by standard MLV components, can be reverse transcribed and integrated by the MLV machinery following infection or transduction of a cell, and will allow Tat-inducible expression of a therapeutic gene from the transduced vector. The basic components of such a vector are as follows (Figure 8):

1. Tat-inducibility. The Tat-inducibility of the HIV LTR promoter is a property of sequences in the U3 region of the promoter and also the TAR element in R (Berkhout and Jeang, 1992). In addition, some other promoters, including the U3 regions from HIV-2 and RSV can substitute for the HIV-1 U3 regions to allow Tat transactivation (Liu *et al.* 1994). In order

for the U3 element to appear in the 5' LTR following reverse transcription, it must be present in the 3' LTR of the viral RNA. The vector therefore contains the HIV U3 and R sequences at the 5'LTR.

2. Reverse transcription. MLV RT initiates reverse transcription at the primer binding site (PBS). This initial (-) strand synthesis extends into U5 and R sequences, forming the first 'strong stop' DNA strand. The RNAseH moiety of RT then degrades the RNA in this hybrid, allowing the exposed DNA to hybridise with the homologous R region in the 3' LTR of the provirus. The homology between the 5' and 3' R regions enables the polymerase to switch strands and continue synthesis along the (-) strand from the 3'LTR. (+) strand DNA synthesis is primed by the selective retention of an RNA fragment at the polypurine tract after RNAse degradation of the genomic RNA strand (reviewed in Katz and Skalka, 1994). The minimum requirements for MLV pol directed reverse transcription contained in the vector are therefore the PBS to initiate (-) strand DNA synthesis, the PPT to initiate (+) strand DNA synthesis and identical 5' and 3' R sequences to allow the first template switch. The requirement for identical R sequences is met by having HIV R sequences in both 5' and 3' LTRs. In addition, as there is evidence to suggest that secondary structures in the 5' U5 region are also important for the initiation of reverse transcription (Cobrink *et al.* 1991), we have kept the MLV U5 sequences in the 5'LTR. The 3' U5 sequences do not appear in the genomic RNA transcript; however, to ensure correct termination at the 3' R/U5 border during genomic transcription, we will use the HIV U5 region in the 3' LTR.

3. Integration. The termini of the reverse-transcribed molecule contain short, sometimes imperfect, inverted repeats of 2-23 bp, which the retroviral integrase recognises (reviewed in Katz and Skalka, 1994). For

MLV, it has been demonstrated that only 9 bases at the end of a linear model substrate are sufficient for almost wild-type levels of integration in an in vitro integration assay system (Bushman and Craigie, 1990). These sequences are derived from the ends of the 3'U3 region and the 5'U5 region in the vector. This requirement is met by the vector containing 36 bases of MLV sequence at the 5' end of the 3' U3 and the whole of the MLV 5' U5 region.

4. Packaging components. Efficient packaging of a vector genome into a retroviral particle is dependent on a number of cis-acting sequences (reviewed in Linial and Miller, 1990). The most important sequence is the packaging signal, a highly structured region of RNA at the 5' region of the genome. In addition, other regions of the genome have been found to increase the efficiency of packaging, including sequences in gag p15. This region is included in standard MLV retroviral vectors, such as LXSN (Miller and Rosman, 1989) and is also preserved in our constructs.

5. High titer retrovirus stocks following transient transfection. We have recently devised a system for the rapid production of high titer retroviral vectors (10^7/ml) by transient transfection (Soneoka *et al.* 1995). A key feature of this system is that the powerful CMV promoter drives high level expression of the vector RNA in the producer cell line and is positioned so that the transcription start site of the vector RNA is exactly the same as the normal LTR-directed start site. To achieve this, we have placed the CMV promoter up to its transcriptional start site adjacent to the start of the HIV R region in the 5' LTR. This principle could be applied to other heterologous promoters, as long as the integrity of the retroviral transcription unit is maintained.

This system has been described for hybrid MLV-HIV vectors, but the same principle can be applied to other combinations of

retroviruses, where one retrovirus is donating the cis-acting sequences required by its own packaging components provided in trans (e.g. SNV, RSV. ASLV etc.) and the other retrovirus is used because of the property of conditional expression of its LTR promoter. Further examples of such retroviral promoters are the HTLV-1 promoter (dependent on Tax protein) and the steroid-hormone inducibile MMTV LTR (reviewed in Majors, 1990). The use of HTLV-1 LTR, for example, to direct expression of a suicide gene could find applications as a treatment for adult T-cell leukemia.

ADVANTAGES OF THE VECTORS DESCRIBED HEREIN

1. Reduced basal levels of expression of therapeutic genes leading to reduced toxicity and immune recognition of target cells.
2. Stricter dependency upon HIV infection to activate gene expression
3. Reduced availability of RNA carrying the packaging site leading to lower risk of inadvertent packaging of the RNA in the target cell.

EXAMPLES

I. Construction of a universal TIN vector (pTIN511) (Figure 2)

The starting molecule is pTIN500 (described in PCT/GB96/01230). This was derived from pTIN414 described in PCT/GB96/01230 (and described herein in detail below and illustrated in Figure 7). PTin414 is the TIN vector equivalent of retroviral vector PHIT111 (Soneoka *et al* 1995), a derivative of LZSN (Adam *et al* 1991). Subsequent TIN vectors were derived from pTIN414 by replacement of internal sequences between unique SpeI and NheI sites. The SpeI site is located within the non-translated gag coding region upstream of the lacZ gene and the Nhe I site is in the 3' U3 region at the junction of the MLV and HIV-1 sequences. Plasmid pTIN500 contains the SpeI - NheI internal

fragment from pBABEpuro. The SV-Puro cassette is deleted by digestion with AccI and religation. This produces pTIN510. A poly-linker is inserted into the unique EcoRI site in pTIN510.

Polylinker sequence (SEQ ID NO: 1)

EcoRI SalI XhoI BglII EcoRI
5'AATTCGTCGACCTCGAGATCCG;
GCAGCTGGAGCTCTAGGCAATT 5'

This creates pTIN511 which has unique SalI, XhoI and BglII sites for the insertion of additional sequences.

The removal of the SV-Puro cassette is not critical for the current invention but serves to simplify the structure of the vector. There may be situations, obvious to one skilled in the area, when the retention of this or any additional cassette might be desirable.

Detailed construction of TIN414.

The co-ordinates of the sequences derived from the CMV promoter, the MLV vector LZSN (Adam *et al.* 1991) and the HIV-1 proviral clone WI3 (Kim *et al.* 1989) are indicated. The molecule was created using standard recombinant DNA techniques and in addition, recombinant PCR to create exact junctions between the different parts of the molecule (Higuchi 1990). Specifically

(i) Construction of the 5' CMV driven LTR

Plasmid pPE611 (Braddock *et al.* 1989) contains the human CMV promoter (from -521 to +1) joined exactly to the start of the HIV-1 R region (co-ordinates +1 to +80). An XbaI - BamHI fragment from this plasmid was ligated into the cloning vector pBluescript (Stratagene) to give

plasmid pRV404. A PCR amplification was performed using plasmid pLNSX as the template, using primers 5'-gcgagctagcttcgaatcgtggtctcgctgttccttgg-3' and 5'ggccgctagcgttcagaactcgtcagttccaccac-3'. The PCR product so generated was digested with NheI and ligated into pRV404 at its NheI site to give plasmid pRV405. Two oligonucleotides of sequence 5'-ttaagcctcaataaagcttgccttgagtgcttcatc-3' and 5'-cggatgaagcactcaaggcaagctttattgaggc-3' were annealed together to create a short duplex containing single stranded regions at either end corresponding to the overhangs present on AflII and BstBI restriction fragments. This molecule was ligated into plasmid pRV405 cut with AflII and BstBI, to give plasmid pRV406.

(ii) Construction of the 3' LTR

A HindIII - XbaI fragment from pLNSX was ligated into the cloning vector pSP72 to give plasmid pRV400. Plasmid pBX+ contains a BamHI - XbaI fragment from WI3 containing the whole 3' LTR from the HIV-1 genome. PCR amplification was performed on pBX+ using primers 5'-ccgcgctagcgatatccttgatctgtggatctaccac-3' and 5'gcgagggtaccgtcgactgctagagattttccacactgac-3'. The PCR product was digested with KpnI and NheI and ligated into plasmid pRV400 digested with KpnI and NheI, to give plasmid pRV401. The ClaI - KpnI fragment of pRV401 was ligated into pBluescript digested with ClaI and KpnI, to give plasmid pRV408.

(iii) Addition of internal sequences.

A SacII - SpeI fragment from pRV406 was ligated into plasmid pRV408 to create plasmid pRV412. The SpeI -NheI fragment from pRV412 was replaced with the SpeI - NheI fragment from LZSN to give vector pTIN414 (Figure 7).

2. Construction of the RRE splice acceptor cassette (RAC) Figure 3.

The starting molecule is pWI3 (Kim *et al* 1989). This contains a full length proviral clone of isolate HIV-1 IIIb clone HXB2 (Ratner *et al* 1985; Genbank accession no.K03455). Coordinates refer to nucleotide positions in the proviral DNA starting with the first nucleotide of the 5' LTR as 1 and are given in brackets following the restriction site. To facilitate cloning steps a subclone is created that contains only the envelope region of HIV-1. To achieve this a SalI (5785) to BamHI (8473) fragment from WI3 is inserted into the polylinker of the cloning vector pSP46 (Promega) to produce plasmid pPE531. The RRE splice acceptor region (RAC) is further subcloned as a BglII (7620) to EcoRI (site in the pSP46 polylinker) into the polylinker site of pSP71 to create pRAC.

3. Construction of pTRAC (Figure 4)

The BglII(7620) to BamHI (8473) fragment is inserted into the unique BglII site in pTIN511. In the correct orientation the upstream BglII site is preserved and the downstream site is a hybrid BglII/BamHI site which is non-functional for either enzyme. pTRAC therefore retains three unique cloning sites upstream of the RAC .

4. Construction of pTRAC-TG (Figure 5)

A coding sequence of choice is inserted into one of the unique restriction sites in pTRAC. This sequence is a therapeutic gene or a reporter gene. The sequence is prepared with appropriate restriction at the termini and has an ATG codon for translation initiation. In the present example the RevM10 sequence is amplified from plasmid pM10 (Malim *et al* 1989a) using PCR primers incorporating flanking BglII (upstream) and BamHI (downstream sites).

Primer sequences:

GGCAGATCTATGGCAGGAAGAAGCGG - 3' (SEQ ID NO: 2)
GGCGGATCCTTCTTTAGTTCCTGACTCC - 3' (SEQ ID NO: 3)

The amplified product is digested with BglI and BamHI and the product is ligated into the unique BglII site of pTRAC. The vector genome is renamed according to the therapeutic gene in this example pTRAC-TG becomes pTRAC-RevM10.

Insertion of the gene in the correct orientation preserves the upstream BglII site but the downstream site is destroyed by the formation of a BglII/BamHI hybrid site.

5. Construction of pTRIN and pTRIN-TG (Figure 6)

To construct an RRE cassette, a 359 base pair fragment encompassing the minimal fully functional RRE is amplified from pRAC using primers that locate with respect to the HIV proviral sequence coordinates at nucleotide 7705 and 8067 (or from pPE351 sequences from 7707 to 8066). The upstream primer adds an EcoRI site followed by a BamHI site and the downstream primer adds an EcoRI site.

Primer 1 (SEQ ID NO: 4) (lower case is HIV-1 sequence 7705 to 7725)
5'CCGCGAATTCGGATCCaggagtagcacccaccaaggc

Primer 2 (SEQ ID NO: 5) (lower case is HIV-1 sequence from 8067 to 8047)
5'CCGCGAATTctccaactagcattccaaggc

The amplified product is digested with EoRI and is ligated into the EcoRI site of pTIN510 to produce pTRIN. This now has a unique BamHI site for the insertion of any additional sequences.

A therapeutic gene or reporter gene is inserted into the unique BamHI site of pTRIN. In the present example the RevM10 cassette described above is inserted. The vector is renamed according to the therapeutic gene in this example pTRIN-TG becomes pTRIN-RevM10.

6. Analysis of pTRAC-TG and pTRIN-TG

Retroviral vector stocks are produced either by transient transfection of 293T cells according to methods of Soneoka *et al* l995 or by the creation of producer cell lines by standard methods e.g. (Cosset *et al* 1995). MLV packaging components are provided in trans on two plasmid components - a gag-pol expression plasmid (pHIT60, an MLV gag-pol expression plasmid described in Soneoka *et al* 1995) and an amphotropic envelope expression plasmid (pHIT456 derived from plasmid SV-A-MLV-env described in Page *et al* 1990 and essentially the same as the ecotropic expression construct pHIT123 described in Soneoka *et al* 1995). Rev is provided in trans from a CMV rev expression plasmid. The following vector genomes are used:-

a) standard MLV vector (pHITlll; Soneoka *et al* 1995)
b) pTRIN-RevM10
c) pTRAC-RevM10
d) pTRIN-lacZ
e) pTRAC-lacZ
f) pTIN414 (as described in PCT/GB96/01230) and shown in Figure 7.

The following additional plasmids are used in the analysis:-

g) pTAT (A CMV Tat expression plasmid as described by Braddock *et al* 1989)
h) pHCMVsrev (A Rev expression plasmid as described by Benko *et al* 1990).

Virus stocks are used to infect HeLa-CD4 cells and human U937 cells. In some cases repeat transfections are done as described by Cannon *et al* (1996). In this procedure the target cells are exposed to fresh retroviral vector stocks every 48 hours. Forty-eight hours after the final transduction the transduced cell populations are infected with HIV-1. After 48 and 72 hours cells are harvested and protein extracts are prepared by standard procedures and assayed for the expression of the marker or therapeutic gene. In addition viral spread through the culture is assessed by determining reverse transcriptase activity in culture supernatants every three days.

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CLAIMS

1. A retroviral vector particle comprising a packagable RNA genome capable of being inserted into a target cell genome when in the form of a DNA provirus, said RNA genome carrying sequences which provide in the DNA provirus at least one selected gene capable of being expressed in the target cell and located within an intron in a transcription unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor.

2. The retroviral vector particle according to claim 1, wherein the polynucleotide response element is responsive to a transactivating retroviral nucleus to cytoplasm transport factor.

3. The retroviral vector particle according to claim 2, wherein the polynucleotide response element is responsive to HIV Rev or a functional equivalent thereof.

4. The retroviral vector particle according to any one of claims 1 to 3, wherein the polynucleotide response element is the Rev response element (RRE) or a function equivalent thereof.

5. The retroviral vector particle according to any one of claims 1 to 4, wherein a selected gene is a therapeutic gene.

6. The retroviral vector particle according to any one of claims 1 to 5, based on an oncoretrovirus.

7. The retroviral vector particle according to any one of claims 1 to 6, based on murine leukemia virus (MLV).

8. The retroviral vector particle according to any one of claims 1 to 7, wherein the 5' long terminal repeat (LTR) of the provirus comprises HIV U5 and R regions or functional portions thereof having Tat inducible

promoter activity, in place of the 5' LTR promoter function of the retrovirus on which the vector particle is based.

9. The retroviral vector particle according to any one of claims 1 to 8, wherein the packaging signal is contained within the intron in which the selected gene is located.

10. A DNA construct encoding the packagable RNA genome for the retroviral vector particle according to any one of claims 1 to 9, operably linked to a promoter.

11. The DNA construct according to claim 10, wherein the promoter is a strong promoter such as the CMV promoter.

12. The DNA construct according to claim 10 or claim 11, wherein the selected gene is absent and the construct has an insertion site within the intron at which the selected gene or genes may be inserted.

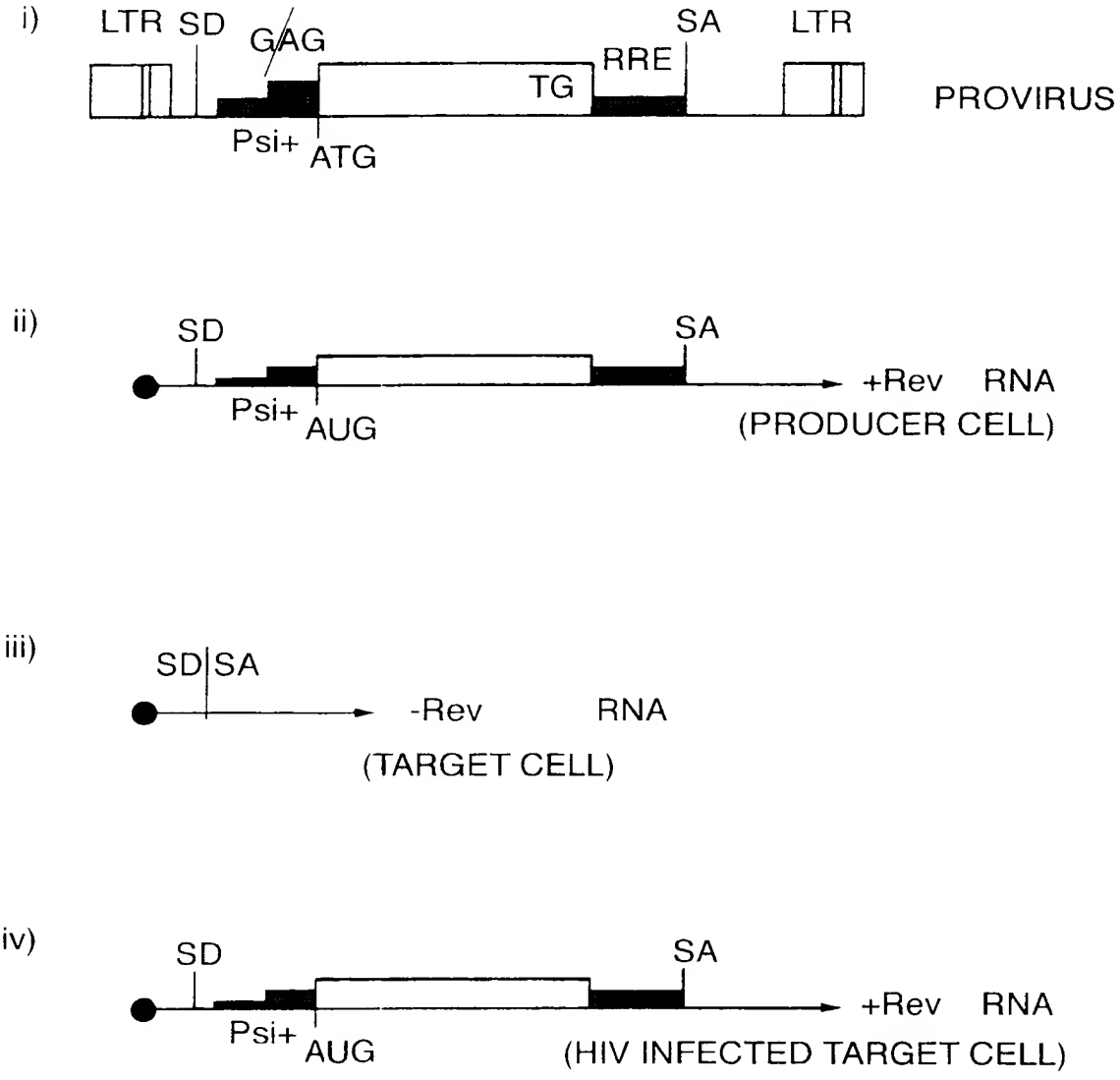
13. A retroviral vector particle production system comprising a host cell transfected with the DNA construct according to any one of claims 10 to 12, said system capable of producing retroviral vector particles according to any one of claims 1 to 8.

14. A retroviral vector particle production system comprising a set of nucleic acid sequences encoding the components of a retroviral vector particle according to any one of claims 1 to 9.

15. The use of a retroviral vector according to any one of claims 1 to 9 for gene therapy for infection or transduction of a target cell.

16. Target cells resulting from the method according to claim 15.

Fig.1.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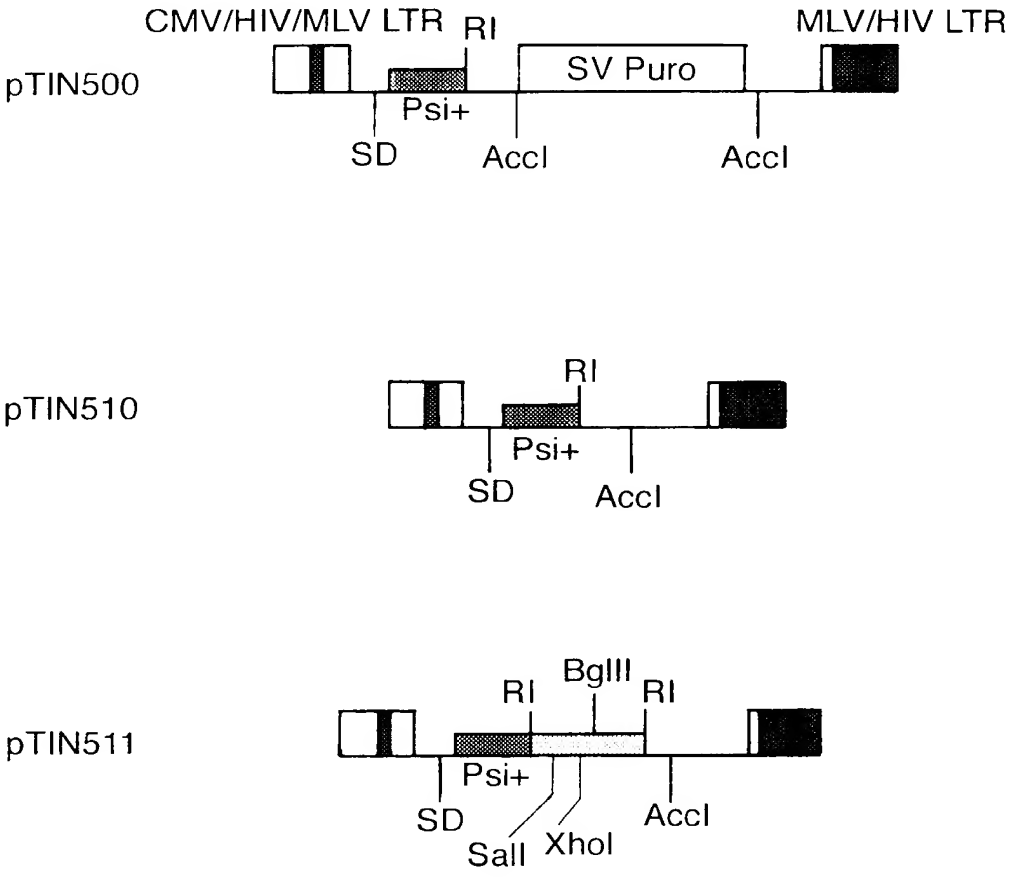
LTR
SD
GAG
SA
LTR
RRE
TG
PROVIRUS
Psi+
ATG
ii)
SD
SA
+Rev RNA
Psi+
AUG
(PRODUCER CELL)
iii)
SD
SA
-Rev RNA
(TARGET CELL)
iv)
SD
SA
+Rev RNA
Psi+
AUG
(HIV INFECTED TARGET CELL)

Fig.2.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RI
MLV/HIV LTR
pTIN500
SV Puro
Psi+
SD
AccI
AccI
pTIN510
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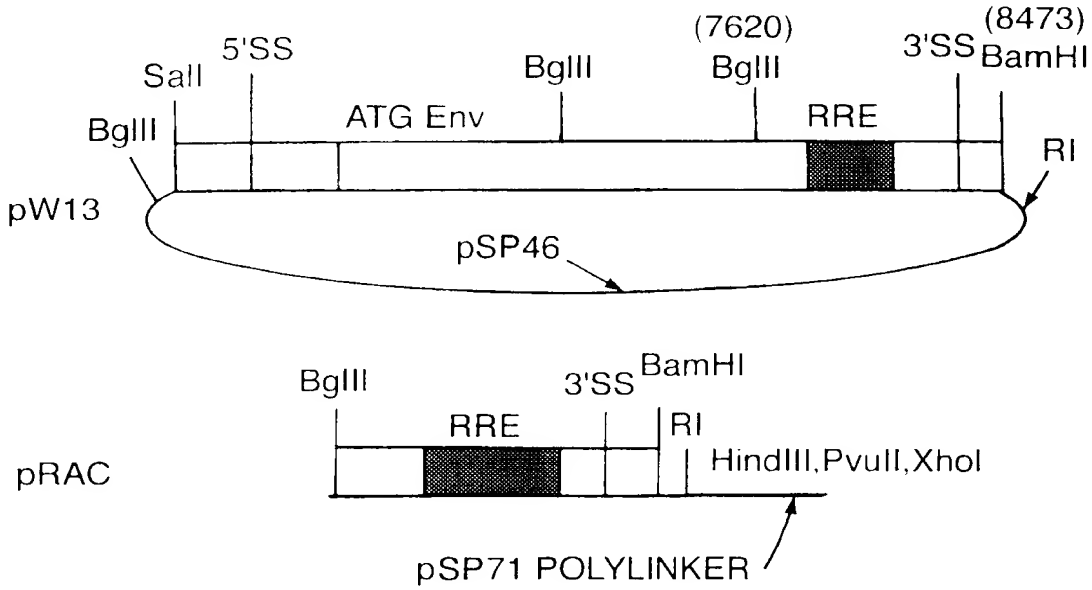
Psi+
SD
AccI
SalI
XhoI

Fig.3.



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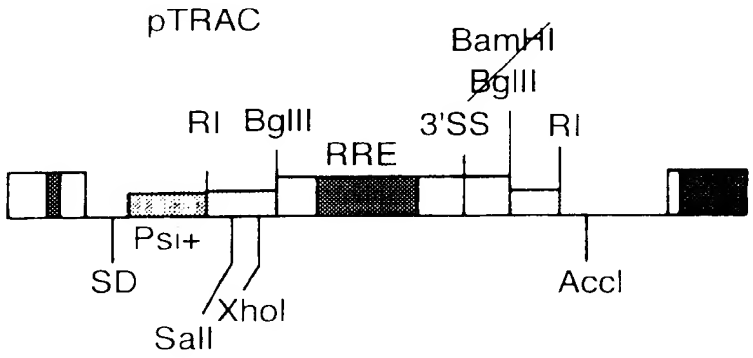


Fig.5.

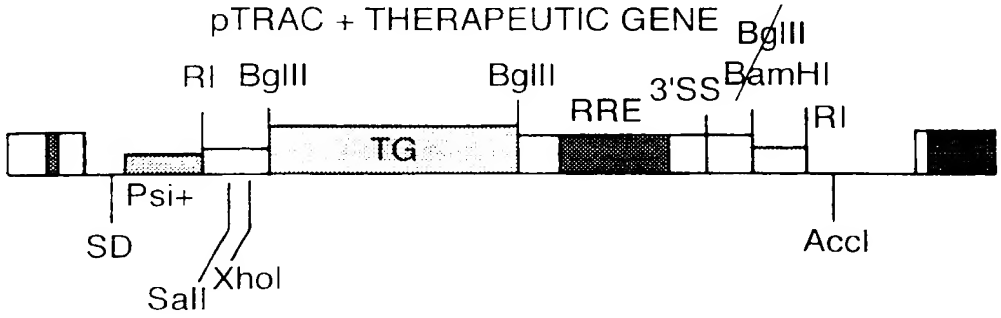
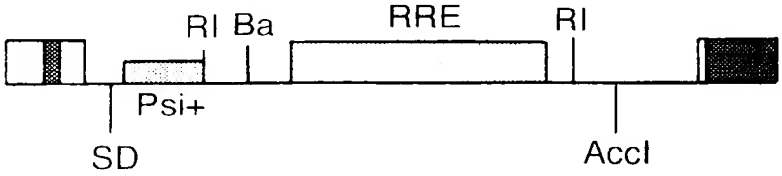


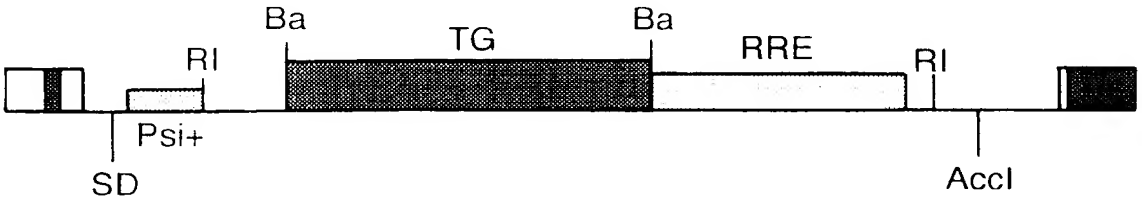
Fig.6.

RRE CASSETTE

pTRIN



pTRIN-TG



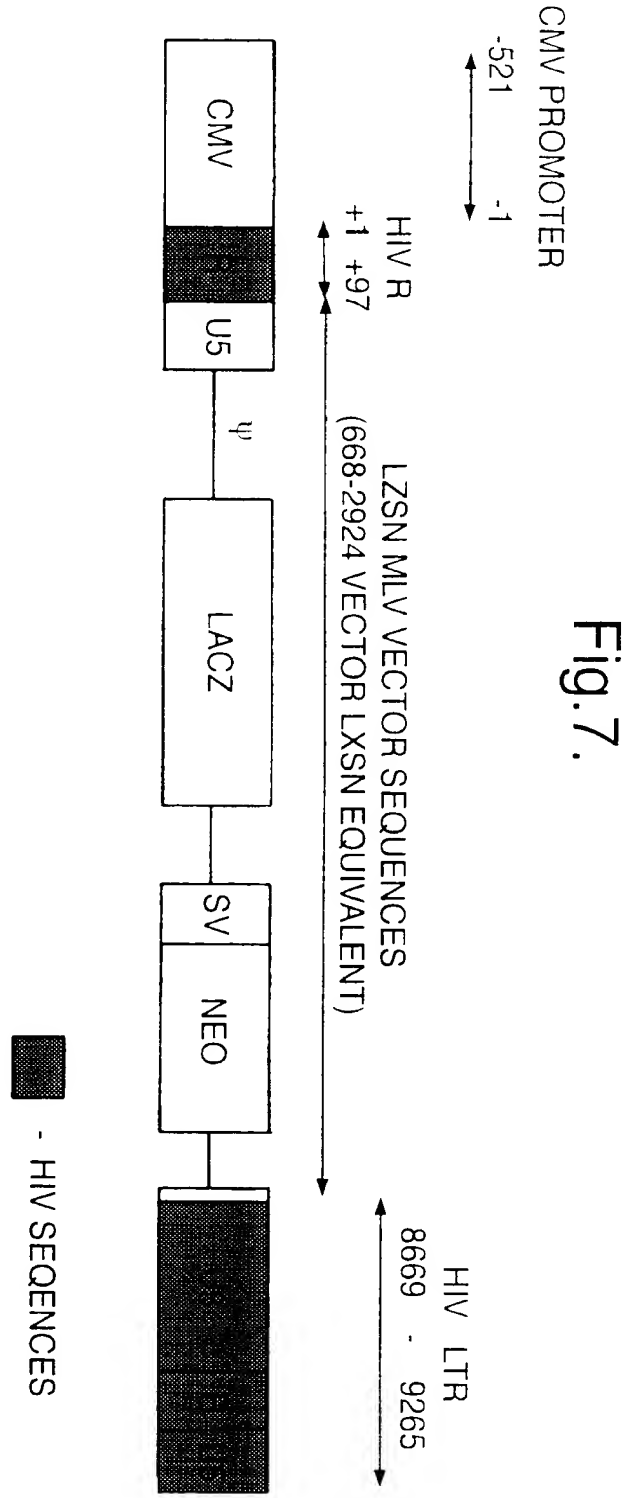
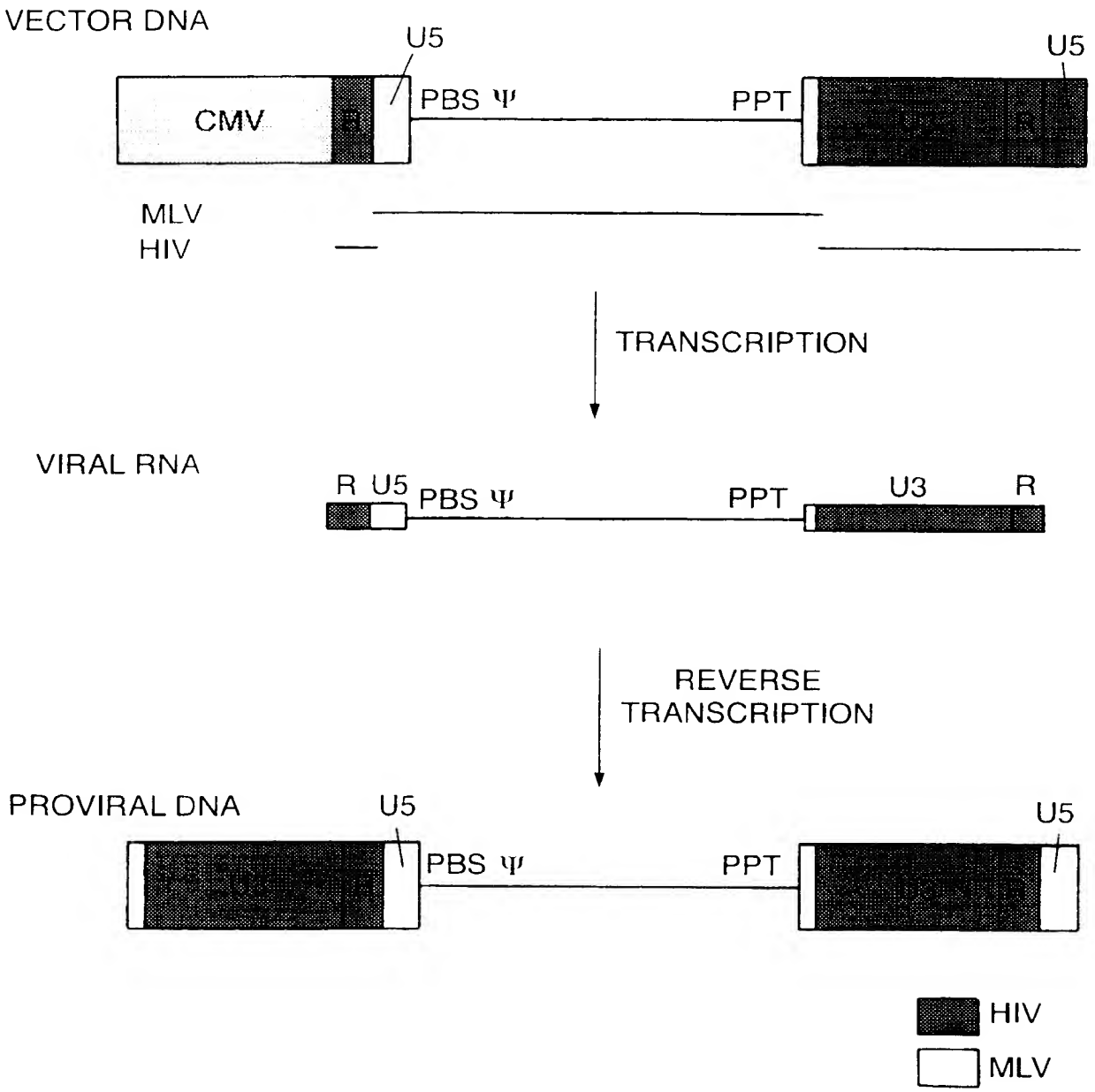

CMV PROMOTER
-521 -1
HIV R
+1 +97
LZSN MLV VECTOR SEQUENCES
(668-2924 VECTOR LXSN EQUIVALENT)
HIV LTR
8669 - 9265
CMV
U5
Ψ
LACZ
SV
NEO
- HIV SEQENCES

Fig.7.

Fig.8.


VECTOR DNA
U5
U5
CMV
R
PBS Ψ
PPT
R
MLV
HIV
TRANSCRIPTION
VIRAL RNA
R U5
PBS Ψ
PPT
U3
R
REVERSE
TRANSCRIPTION
PROVIRAL DNA
U5
U5
PBS Ψ
PPT
HIV
MLV

INTERNATIONAL SEARCH REPORT

International Application No
PC./GB 97/02859

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 C12N7/00 A61K48/00 C07K14/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 21750 A (US GOVERNMENT) 10 December 1992 see page 8, paragraph 5 - page 9, paragraph 3 see page 14 - page 16 see page 12, paragraph 2 - page 13, paragraph 3 ---	1-16
Y	HOPE, T.J. ET AL.: "Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE OF THE USA, vol. 87, October 1990, pages 7787-7791, XP002056927 see the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 1998	17. 03. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

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INTERNATIONAL SEARCH REPORT

Inte[illegible]ional Application No
PCT/GB 97/02859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	MCLACHLIN J R ET AL: "RETROVIRAL-MEDIATED GENE TRANSFER" PROGRESS IN NUCLEIC ACID RESEARCH AND MOLECULAR BIOLOGY, vol. 38, 1990, pages 91-135, XP002030826 see page 119 ---	1-16
A	US 5 306 631 A (HARRISON GAIL ET AL) 26 April 1994 see claims 4,8,11; example 4 ---	1-16
A	WO 95 30755 A (HISAMITSU PHARMACEUTICAL CO ;SHIMADA TAKASHI (JP); AKIYAMA KATSUHI) 16 November 1995 see figure 1 & EP 0 759 471 A -----	11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB 97/02859

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos..
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 97/02859

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 15 is directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.